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In Vivo Mechanisms of Natural Killer Cell Tolerance

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by
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ABSTRACT OF THE DISSERTATION

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Natural killer (NK) cells are cytotoxic innate immune cells that provide protection from pathogens and tumors. To carry out these functions, NK cells must distinguish between healthy and unhealthy self-cells. Inability to recognize stressed cells would lead to a failure of NK-cell immunity whereas inability to identify healthy cells could lead to NK-cell autoimmunity. It remains unclear, however, how NK cells are able to distinguish healthy and unhealthy self-cells with a limited repertoire of germline-encoded receptors. The "missing-self" hypothesis proposes that NK cells identify stressed cells by their reduced expression of MHC class I (MHC-I) that is almost ubiquitously expressed as self. NK cells express inhibitory Ly49 receptors that bind to MHC-I and inhibit NK cells from killing healthy cells, and downregulation of MHC-I on stressed cells leads to loss of inhibition and killing by missing-self recognition. The importance of Ly49 receptors and MHC-I for maintaining NK-cell self-tolerance, however, has only been suggested by *in vitro* experiments and correlative *in vivo* experiments. Here we generated a mouse with a mutation in the immunoreceptor tyrosine-based inhibitory motif of Ly49A and another mouse with an allele of the gene for beta$_2$-microglobulin containing loxP sites (*B2m*^{fl}) to

directly test the roles of Ly49s and MHC-I in NK cell self-tolerance *in vivo*. Loss of inhibitory signaling through a mutant Ly49 or global MHC-I downregulation induced changes in NK-cell responsiveness or inhibitory receptor expression that maintained NK-cell self-tolerance. In contrast, downregulation of MHC-I on $CD4^+$ T cells, led to a subtle loss of $CD4^+$ T cells, but NK cells remained tolerant to a substantial population of MHC-I-deficient $CD4^+$ T cells without altering their responsiveness or receptor repertoire. In this setting, infection with murine cytomegalovirus or treatment with a toll-like receptor agonist induced NK cell-mediated rejection of MHC-I-deficient $CD4^+$ T cells. These results show that loss of inhibitory signaling to NK cells *in vivo* can induce tolerance or rejection of missing-self in different contexts and that inflammation promotes missing-self reactivity.

Chapter 1: Introduction

1.1 Natural killer cells

1.1.1 NK cell definition and function

Innate immune cells are defined by their ability to recognize pathogens or infected cells through germline-encoded receptors (Janeway and Medzhitov, 2002). This is in contrast to adaptive immune cells, such as B and T cells, that require gene rearrangement to form antigen-specific receptors (Janeway and Medzhitov, 2002). Gene rearrangement generates a much larger repertoire of antigen receptors than can be germline-encoded in the genome, which provides adaptive immune cells the ability to recognize a substantially broader array of non-self molecules than innate immune cells (Vivier et al., 2011). How the innate immune system is able to discriminate self from non-self utilizing a narrow repertoire of germline-encoded specificities is an active area of investigation. Because of the large repertoire of adaptive immune receptors, T and B cells expressing a particular antigen receptor are rare and must clonally expand over a period of days prior to providing protective immunity (Janeway and Medzhitov, 2002). In contrast, innate immune cells presumably do not require clonal expansion and are able to respond rapidly to infections, which in part functions to protect the host long enough for the adaptive immune system to respond (Janeway and Medzhitov, 2002).

Natural killer (NK) cells are innate immune cells that were first identified as non-T non-B lymphocytes from the spleens of naïve mice that could lyse the YAC-1 Moloney leukemia virus (MLV)-induced tumor cell line *in vitro* (Kiessling et al., 1975a; Kiessling et al., 1975b). NK cells were labeled as “natural” killers because cytotoxicity was observed without prior immunization

in contrast to T cell-mediated killing, which requires immunization to prime antigen-specific naïve T cells and increase their frequency by clonal expansion (Williams and Bevan, 2007). Natural killer activity was also observed in lymphocytes from the peripheral blood of humans when incubated with the K-562 myeloid leukemia cell line (Jondal and Pross, 1975). NK cells are categorized as innate immune cells due to this ability to kill target cells without prior sensitization (Vivier et al., 2011) and because functional NK cells develop in SCID mice (Dorshkind et al., 1985) and RAG-deficient mice (Shinkai et al., 1992) that lack the ability to rearrange antigen receptor gene segments, which suggests that NK cells utilize germline-encoded receptors.

Consistent with their initially described ability to lyse tumor cells *in vitro*, NK cells protect mice from transplanted and spontaneous tumors *in vivo* (Guerra et al., 2008; Karre et al., 1986; Talmadge et al., 1980). NK cells also reject MHC-mismatched bone marrow transplants (Cudkowicz and Bennett, 1971; Murphy et al., 1987a) and protect mice from infections with intracellular pathogens, such as murine cytomegalovirus (MCMV) (Bukowski et al., 1983). NK cells provide immunity *in vivo* through both direct cytotoxicity and the production of cytokines, such as interferon-γ (IFN-γ) (Long et al., 2013; Presti et al., 1998). The mechanism of NK cell-mediated cytotoxicity is thought to utilize granzyme B (Shresta et al., 1995) and perforin (Lowin et al., 1994) similar to $CD8^+$ T cells (Long et al., 2013) based on *in vitro* assays. As such, perforin- and granzyme B-deficient mice are susceptible to MCMV (Fehniger et al., 2007). However, NK cell-mediated rejection of mismatched bone marrow grafts is independent of perforin (Baker et al., 1995; Bennett et al., 1998), suggesting that differences may exist between the mechanisms of action of NK cells *in vitro* and *in vivo*.

Notably, the originally described "natural" cytotoxicity by fresh NK cells *in vitro* was restricted to a small number of tumor cell targets (Kiessling et al., 1975b; Rosenstein et al., 1984) and was ineffective against most fresh tumor cells from both mice (Kiessling et al., 1975b) and humans (Grimm et al., 1982; Vose and Moore, 1980). Subsequent studies showed that NK cells could kill a substantially broader array of target cells *in vitro* including fresh tumor cells after culture in IL-2 to generate "lymphokine-activated killer" (LAK) cells (Grimm et al., 1982; Rosenstein et al., 1984), by treatment with interferon (Djeu et al., 1979; Vose and Moore, 1980), or by pretreating mice with poly(I:C) (Djeu et al., 1979; Liao et al., 1991). Similarly, NK cell-mediated cytotoxic control of MCMV requires priming of NK cells by type I interferon and IL-12 (Parikh et al., 2015), which suggests that NK cells require cytokine activation to provide protective immunity to viral infection. In contrast, however, NK cells reject tumor cells (Kärre et al., 1986) and MHC-mismatched bone marrow grafts (Cudkowicz and Bennett, 1971; Murphy et al., 1987a) *in vivo* without pre-treating mice with inflammatory stimuli. As a result, it still remains unclear if NK cell-mediated protective immunity is truly "natural" or requires prior activation *in vivo*.

1.1.2 NK cell development

NK cells were initially characterized as lymphocytes based on cellular morphology (Kiessling et al., 1975a). Consistent with NK cells belonging to the lymphoid lineage with T and B cells, mice lacking the transcription factor Ikaros lack all three of these cell populations (Georgopoulos et al., 1994). Additionally, a common lymphoid progenitor (CLP) in the mouse bone marrow gives rise to T, B, and NK cells (Kondo et al., 1997). NK cells, however, develop in athymic nude mice that cannot support T cell development (Kiessling et al., 1975a) as well as SCID mice and RAG-deficient mice as mentioned previously (Dorshkind et al., 1985; Shinkai et

al., 1992). Moreover, bone marrow progenitor populations have been identified that give rise to NK cells but not T or B cells, which separates NK cells as a distinct lineage from T and B cells within the lymphoid lineage (Fathman et al., 2011; Rosmaraki et al., 2001).

After release from the bone marrow, NK cells continue to progress through stages of "maturation" that are delineated based on expression of CD11b and CD27 (Hayakawa and Smyth, 2006; Kim et al., 2002). NK cells progress from immature $CD11b^-$ $CD27^-$ cells to $CD11b^-$ $CD27^+$ to $CD11b^+$ $CD27^+$ to terminally mature $CD11b^+$ $CD27^-$ (Chiossone et al., 2009). Mature NK cells exhibit increased or decreased functionality compared to immature NK cells depending on the particular study (Hayakawa and Smyth, 2006; Joncker et al., 2010; Kim et al., 2002). However, knockout mice that exhibit blocks in NK cell maturation, also exhibit defects in NK cell-mediated tumor rejection (Gordon et al., 2012; van Helden et al., 2015; Werneck et al., 2008). As a result, NK cell maturation status is often used as metric of NK cell functionality. Notably, however, the stages of NK cell development and maturation do not correspond to any known steps of positive or negative selection such as those that establish central tolerance during T cell development in the thymus (Xing and Hogquist, 2012).

1.2 The "missing-self" hypothesis

1.2.1 NK cells recognize "missing-self"

MHC-I is a nearly ubiquitously expressed cell surface protein complex composed of a polymorphic heavy chain (H-$2K^b$ or H-$2D^b$ in C57BL/6 mice) and $beta_2$-microglobulin (B2m) that presents intracellular peptides to cytotoxic $CD8^+$ T cells (Neefjes et al., 2011). Intracellular pathogens and transformed cells evolve strategies to downregulate MHC-I to evade clearance by MHC-I-restricted cytotoxic T cells (Malmberg et al., 2017). In response, NK cells are thought to

recognize and kill target cells that downregulate MHC-I through "missing-self" recognition to complement cytotoxic T cell-mediated immunity (Kärre et al., 1986; Ljunggren and Karre, 1990). As a result, the missing-self hypothesis provides a theoretical framework for how NK cells can recognize stressed cells with a limited number of germline-encoded receptors.

Initial evidence for the missing-self hypothesis came from experiments showing that NK cells reject the MHC-I-deficient tumor cell line RMA/s but not the MHC-I-positive tumor cell line RMA after adoptive transfer *in vivo* (Kärre et al., 1986). Additionally, NK cells from poly(I:C)-treated mice kill RMA/s but not RMA cells *in vitro* (Kärre et al., 1986). Subsequent studies showed that NK cells from H-2D^{d}-transgenic (D8) mice reject non-transgenic bone marrow, which suggested that missing-self recognition also explained the previously recognized ability of NK cells reject MHC-mismatched bone marrow grafts (Murphy et al., 1987b; Ohlen et al., 1989).

Definitive evidence for the missing-self hypothesis, however, came only after the development of the *B2m* knockout (*B2m*$^{-/-}$) mouse that lacks surface MHC-I expression altogether (Koller et al., 1990; Zijlstra et al., 1990). NK cells from wild type (WT) mice reject adoptively transferred *B2m*$^{-/-}$ bone marrow and splenocytes *in vivo* (Bix et al., 1991; Oberg et al., 2004). Additionally, NK cells from mice pre-treated with poly(I:C) or tilorone directly kill concanavalin A (ConA)-induced *B2m*$^{-/-}$ T cell blasts *in vitro* (Hoglund et al., 1991; Liao et al., 1991). For unclear reasons, however, *B2m*$^{-/-}$ mice do not exhibit NK cell-mediated autoimmunity (Liao et al., 1991). As a result, evidence for the missing-self hypothesis is restricted to *in vitro* experiments with pre-activated NK cells and *in vivo* experiments using adoptively transferred MHC-I-deficient targets.

1.2.2 Inhibitory receptors mediate "missing-self" recognition

Inhibitory Ly49 receptors are thought to be the missing-self receptors in mice. Inhibitory Ly49s directly bind to MHC-I to inhibit killing of healthy cells that express normal levels of self-MHC-I (Karlhofer et al., 1992). As a result, downregulation of MHC-I on target cells is thought to induce NK cell activation and killing through a loss of inhibitory Ly49 signaling (Karlhofer et al., 1992). Humans express a structurally divergent family of receptors called the killer immunoglobin-like receptors (KIRs) that also bind to MHC-I to mediate missing-self recognition through an analogous mechanism (Colonna and Samaridis, 1995; Long et al., 2013; Moretta et al., 1993).

Ly49s form a family of activation and inhibitory receptors encoded by the *Ly49/Klra* genes within the NK gene complex (NKC) on mouse chromosome 6 (Yokoyama and Plougastel, 2003). The inhibitory Ly49s expressed by NK cells in C57BL/6 (B6) mice are Ly49A, Ly49C, Ly49F, Ly49G2, and Ly49I (Orr and Lanier, 2011). All of these inhibitory Ly49s have been shown to bind to MHC-I with gene- and allele-specificity (Orr and Lanier, 2011). Importantly, H-2K^{b} from B6 mice is a ligand for Ly49C and Ly49I (Hanke et al., 1999; Scarpellino et al., 2007). In contrast, H-2D^{d} is a ligand for Ly49A, Ly49C, Ly49G2, and Ly49I (Hanke et al., 1999). Ly49F promotes adhesion to target cells of the H-2^{d} haplotype, but the exact ligand is unknown (Hanke et al., 1999). Importantly, most ligands for Ly49s have been identified using tetramer-binding and cell-adhesion assays with sometimes inconsistent results (Hanke et al., 1999; Scarpellino et al., 2007). Additionally, the binding affinities and functional significance of many of these Ly49-MHC-I interactions have not been assessed. As a result, the physiological ligand for many inhibitory Ly49 receptors remains unclear.

All inhibitory Ly49 receptors are thought to signal through a single cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM) with the consensus sequence (I/L/V/S)xYxx(L/V), which is the only known Ly49 signaling motif (Orr and Lanier, 2010). Binding between Ly49s and MHC-I induces phosphorylation of the tyrosine within the Ly49 ITIM, which then recruits the phosphatases SHP-1, SHP-2, and SHIP that interfere with the activity of kinases that function downstream of activation receptor signaling (Nakamura et al., 1997; Olcese et al., 1996; Wang et al., 2002).

The function of Ly49 receptors was initially identified by showing that antibody blockade of Ly49A relieved inhibition of LAK cells *in vitro* to promote killing of H-2D^{d+} tumor cells (Karlhofer et al., 1992). The *in vivo* functions of Ly49 receptors, however, are poorly understood. Antibody depletion experiments have suggested that Ly49C/I^{+} NK cells reject bone marrow that lacks H-2^{b} and Ly49A/G2^{+} NK cells reject bone marrow that lacks H-2^{d}, but these studies are confounded because antibody depletions of Ly49 subsets reduce the total number of NK cells and because Ly49 expression may correlate with rather than cause missing-self recognition (Brennan et al., 1996; Sentman et al., 1989; Sun et al., 2012). Additionally, the role of the Ly49 ITIM in effector inhibition has only been shown with a transfected rat NK cell line and with primary NK cells from a mouse expressing an ITIM-mutant Ly49A^{BALB} transgene (Ly49A-Y8F^{BALB}) (Chalifour et al., 2009; Nakamura et al., 1997). Definitive studies of Ly49s could be performed with mice carrying targeted mutations in endogenous *Ly49* genes, but technical challenges associated with targeting a specific member of a polymorphic multigene family have prevented the generation of *Ly49* mutant mice.

1.2.3 Unexplained features of "missing-self" recognition

Initial studies of *B2m*$^{-/-}$ mice revealed that NK cells from MHC-I-deficient mice are surprisingly not autoreactive as predicted by the missing-self hypothesis (Bix et al., 1991; Liao et al., 1991). NK cells from *B2m*$^{-/-}$ mice are instead unable to reject MHC-I-deficient bone marrow grafts *in vivo* and are ineffective at killing MHC-I-deficient ConA T cell blasts *in vitro* (Hoglund et al., 1991; Liao et al., 1991). These findings suggest that NK cells from *B2m*$^{-/-}$ mice establish tolerance to missing-self through unknown mechanisms. As a result, the requirements for missing-self recognition *in vivo* are unclear.

NK cells from 50:50 mixed WT:*B2m*$^{-/-}$ fetal liver chimeras are also tolerant of adoptively transferred MHC-I-deficient bone marrow and splenocytes similar to *B2m*$^{-/-}$ mice (Shifrin et al., 2016; Wu and Raulet, 1997). Intriguingly, MCMV infection or cytokine treatment breaks tolerance to missing-self in WT:*B2m*$^{-/-}$ chimeras, leading to a reduction in the percentage of *B2m*$^{-/-}$ cells that contribute to chimerism (Shifrin et al., 2016; Sun and Lanier, 2008a). In contrast, NK cells from WT mice reject adoptively transferred MHC-I-deficient tumor cells and splenocytes without pre-activation with cytokines or infection (Kärre et al., 1986; Oberg et al., 2004). As a result, it is unclear if cytokines are only required to overcome tolerance to missing-self that develops from chronic exposure to MHC-I-deficient cells in mixed chimeras or if cytokines are more generally required for missing-self *in vivo*.

1.3 NK cell self-tolerance

1.3.1 Receptor repertoire model of NK cell tolerance

NK cells are thought to discriminate between healthy and unhealthy cells through germline-encoded activation and inhibitory receptors (Long et al., 2013). Each NK cell expresses a repertoire of numerous activation and inhibitory receptors that bind to a combination of host-

and pathogen-encoded ligands expressed on the surface of host cells (Long et al., 2013). As a result, NK cells decide whether to kill or remain tolerant to a cell based on the balance of signals through multiple receptors (Long et al., 2013). Importantly, many of these activation and inhibitory receptors are only expressed on a fraction of NK cells in a manner that is commonly thought to be stochastic (Raulet et al., 2001). As a consequence, NK cells form a heterogeneous population composed of a large repertoire of related cells that express different combinations of activation and inhibitory receptors, presumably leading to numerous specificities (Raulet et al., 2001). Mass cytometry has been used to estimate that the human NK cell pool within an individual is composed of 6,000 to 30,000 subpopulations based on variegated expression of surface receptors (Horowitz et al., 2013).

T cell development in the thymus involves positive selection for T cells that recognize self-peptide-MHC with intermediate affinity and negative selection to delete T cells that recognize self-peptide-MHC too strongly (Xing and Hogquist, 2012). These processes are thought to generate a repertoire of T cells that recognize self-MHC but are self-tolerant (Xing and Hogquist, 2012). Early studies on human NK cell clones suggested that receptor repertoire selection may also establish NK cell self-tolerance by ensuring that each NK cell expresses at least one self-MHC-I-specific inhibitory receptor (Valiante et al., 1997). Consistent with the receptor repertoire model of NK cell tolerance, NK cells from mice with different MHC haplotypes express altered inhibitory Ly49 repertoires (Held et al., 1996b). Additionally, NK cells from Ly49A transgenic mice exhibit reduced coexpression of the H-2D^d-specific receptors Ly49A and Ly49G2 on an H-2^d background, which suggests selection against NK cells that express too many self-MHC-I-specific inhibitory receptors (Hanke et al., 1999; Held and Raulet,

1997). However, the mechanism of receptor repertoire skewing and the role of Ly49 signaling remain unclear.

In opposition to the receptor repertoire model of NK cell tolerance, some NK cells in WT mice do not express any known self-MHC-I-specific inhibitory receptors but remain self-tolerant (Fernandez et al., 2005). Additionally, although NK cells from MHC-I-deficient mice express a skewed repertoire of inhibitory Ly49s (Salcedo et al., 1997), it is unclear how skewed receptor expression could explain self-tolerance in the absence of a self-MHC-I ligand. As a result, it is unlikely that the receptor repertoire development model fully explains NK cell self-tolerance unless NK cells express unidentified receptors that recognize non-MHC self-ligands to prevent autoreactivity.

1.3.2 NK cell education

Multiple studies have suggested that NK cells establish self-tolerance by modulating their functionality through an "education" or "licensing" process instead of by receptor repertoire selection (Fernandez et al., 2005; Kim et al., 2005). As mentioned previously, NK cells from *B2m*$^{-/-}$ mice exhibit impaired *in vitro* killing of MHC-I-deficient ConA blasts (Liao et al., 1991), but it is unclear if this killing defect is due to changes in the receptor repertoire or other mechanisms because all of the relevant receptors are not known. To bypass this issue, platebound antibody-mediated crosslinking has been used to show that NK cells from MHC-I-deficient mice are generally hypo-responsive to stimulation through activation receptors (Fernandez et al., 2005; Kim et al., 2005). NK cells from WT mice that lack inhibitory receptors for self-MHC-I are also hypo-responsive to platebound antibody stimulation (Fernandez et al., 2005). These results have been used to argue that NK cells from MHC-I-deficient mice and NK cells that lack inhibitory receptors for self-MHC-I are self-tolerant because they are uneducated (Yokoyama

and Kim, 2006). However, the *in vivo* significance of NK cell education has not been directly tested.

Transfer of uneducated NK cells from an MHC-I^{-} to an MHC-I^{+} mouse induces acquisition of NK cell education, and the reverse transfer induces loss of NK cell education (Elliott et al., 2010; Joncker et al., 2010). These results have been used to argue that NK cell education is plastic so that NK cells can become tolerant to changing MHC-I environments (Joncker et al., 2010). Notably, however, NK cells from WT mice reject transferred MHC-I-deficient cells (Bix et al., 1991; Oberg et al., 2004) and T cells from *B2m*$^{-/-}$ mice reject transferred WT cells, which could have impacted the outcomes of these transfer experiments (Apasov and Sitkovsky, 1993; Glas et al., 1994; Zijlstra et al., 1992). An inducible MHC-I transgenic mouse was used to show that NK cell education can be acquired without adoptive transfers (Ebihara et al., 2013), but analogous studies have not been performed to test if NK cell education is lost after MHC-I downregulation *in vivo*. Importantly, if NK cells adapt to a loss of MHC-I *in vivo*, then it is unclear when missing-self recognition would occur.

1.3.3 Mechanisms of NK cell education

Two mechanisms have been proposed to explain NK cell education: disarming and arming (Raulet and Vance, 2006). The disarming hypothesis proposes that self-MHC-I-specific inhibitory Ly49s promote NK cell responsiveness indirectly by preventing overactivation of NK cells leading to anergy (Shifrin et al., 2014). The disarming hypothesis is appealing because it only requires Ly49 receptors to function as inhibitory receptors and because it is similar to mechanisms of anergy and activation-induced cell death that control peripheral tolerance of T cells (Xing and Hogquist, 2012). Additionally, chronic exposure to activation ligands in transgenic mice can lead to NK cell hypo-responsiveness (Champsaur et al., 2010; Tripathy et

al., 2008). It is unclear, however, whether chronic stimulation through activation ligands induces a general or receptor-specific hypo-responsiveness (Sun and Lanier, 2008b). Additionally, it is unclear if activation ligands are expressed at steady-state in a WT mouse although recent studies have suggested that NKG2D and SLAM family receptor ligands may be potential candidates (Chen et al., 2016; Thompson et al., 2017).

In contrast, the arming hypothesis proposes that binding between inhibitory Ly49s and self-MHC-I directly promotes NK cell functionality (Yokoyama and Kim, 2006). As a result, the arming model implies that Ly49s signal through currently unidentified pathways that differ from the pathways required for effector inhibition. Binding between Ly49 and MHC-I in *cis* on the NK cell surface has been proposed as a mechanism for arming (Bessoles et al., 2014). Studies using transgenic mice expressing mutant Ly49A that binds to H-2D^{d} in *trans* but not in *cis* suggest that unengaged Ly49A tonically suppresses NK cell function and *cis* binding between Ly49A and H-2D^{d} relieves tonic inhibition to arm NK cells (Chalifour et al., 2009). Evidence that Ly49A binds to H-2D^{d} in *cis* comes from studies showing that the mean fluorescence intensity (MFI) of Ly49A is reduced in mice that express H-2D^{d} but elevated after acid-stripping NK cells to destabilize surface MHC-I (Andersson et al., 2007; Doucey et al., 2004; Karlhofer et al., 1994). However, acid-stripping only increases the MFI of Ly49A when stained with the YE1/48 and not the JR9 monoclonal antibody even though both antibodies show a reduced MFI in H-2D^{d+} mice (Andersson et al., 2007). These results suggest that the downregulation of the Ly49A MFI may be due to signaling-induced receptor internalization instead of or in addition to *cis* binding. Additionally, it is unclear whether other Ly49s in mouse and KIRs in humans bind to MHC-I in *cis*, which makes it unclear if the *cis* model provides definitive evidence for the arming hypothesis. Differentiation between the arming and disarming hypotheses will require

identification of the activation ligands that disarm NK cells or identification of the signaling pathways required for arming.

Notably, these models both assume that Ly49 signaling controls NK cell education. Retroviral bone marrow chimeras and Ly49A-Y8F^{BALB} transgenic mice that express Ly49A with an ITIM mutation have been used to argue that Ly49 ITIM signaling is necessary for NK cell education (Bessoles et al., 2013; Kim et al., 2005). However, definitive studies require the generation of Ly49 mutant mice. Additionally, the downstream phosphatase SHP-1 has been reported to be both dispensable and required for NK cell education depending on the whether a spontaneous SHP-1 mutant or an NK cell-specific SHP-1 knockout mouse was studied (Kim et al., 2005; Viant et al., 2014). As a result, the role of the Ly49 ITIM and downstream signaling pathways in NK cell education remain unclear.

Chapter 2: Immunoreceptor tyrosine-based inhibitory motif–dependent functions of an MHC class I-specific NK cell receptor

Published in the *Proceedings of the National Academy of Sciences of the United States of America* (Bern et al., 2017).

2.1 Introduction

The functions of Ly49 receptors *in vivo* have been studied using retroviral bone marrow chimeras and Ly49A transgenic mice (Chalifour et al., 2009; Kim et al., 2005). However, these experiments, particularly with Ly49 transgenic mice, carry the caveats that the site of transgene insertion is unknown, transgenic Ly49 is expressed at non-physiological levels and times during NK cell development, and on cells other than NK cells. As a result of these caveats, one Ly49A transgenic line has been shown to exhibit a complete block in NK cell development (Kim et al., 2000; Kim et al., 2006), which appears to be inconsistent with studies of WT mice. Thus, the role of Ly49 ITIM signaling in NK cell development, effector inhibition, education, Ly49 receptor expression, and receptor repertoire development remain incompletely understood.

Specific targeted mutations in *Ly49*s have been challenging to generate because the *Ly49* genes are highly related and clustered in the NK gene complex (NKC), the *Ly49* cluster has a high concentration of repetitive elements (Makrigiannis et al., 2005), and the *Ly49* cluster in B6 mice encodes distinct Ly49s from those in the 129-strain initially favored for embryonic stem (ES) cell targeting. Ly49 knockout mice have been successfully generated for only Ly49Q^{129}, which is expressed exclusively on myeloid cells (Tai et al., 2008), and Ly49E, which is expressed exclusively on liver tissue-resident NK cells but not conventional splenic NK cells

(Aust et al., 2011; Sojka et al., 2014). Another attempt with a *Ly49* targeting construct led to generation of the NKC knockdown (NKC^{KD}) mouse that contains a concatemerized targeting construct inserted in the NKC (Belanger et al., 2012). Although the NKC^{KD} mouse was shown to express reduced levels of Ly49s, results from this mouse are confounded because the NKC is derived from the 129-strain, Ly49 expression is not completely lost, and expression of other NKC receptors encoded near the *Ly49* gene cluster are also affected by the concatemer insertion.

In this study, we generated mice with a targeted mutation in *Klra1* (*Ly49a*) within the C57BL/6 NKC. This "AYF" allele encoded Ly49A with a single amino acid mutation predicted to abolish ITIM signaling that was validated. The AYF mouse allowed us to study for the first time the effect of losing endogenous Ly49 ITIM signaling on the *in vivo* development and function of primary NK cells.

2.2 Results

2.2.1 Generation of Ly49A ITIM mutant mice

To investigate the functions of Ly49A ITIM signaling in primary NK cells, we introduced a single nucleotide mutation in exon 4 of the *Klra1* gene encoding Ly49A directly in C57BL/6 ES cells that conferred a tyrosine-to-phenylalanine mutation in the ITIM of Ly49A (Fig. 2.1A). Southern blot analysis verified correct targeting of the *Klra1* locus in 7/370 clones (1.9%) (Fig. 2.1B), and two of these clones were found to have a normal karyotype. Correctly targeted ES cell clones were microinjected into Albino B6 blastocysts. Mice containing the germline-transmitted targeted allele were bred to a CMV-Cre transgenic line to remove the neomycin resistance cassette. The resulting allele is referred to as "AYF" (Fig. 2.1A). AYF homozygous mice were found to contain a normal percentage and number of $Ly49A^{+}$ NK cells (Fig. 2.2A-C).

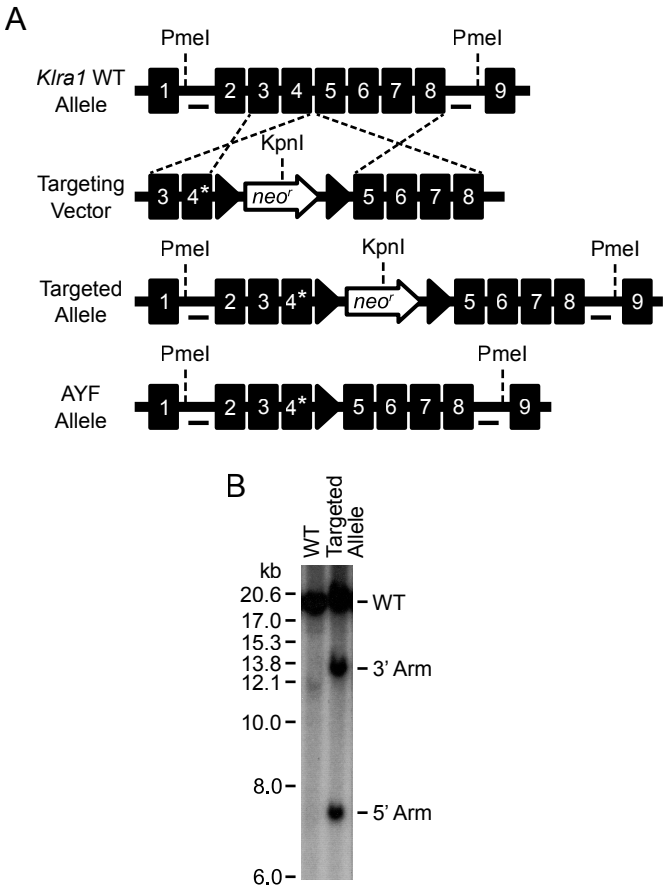


Figure 2.1: Generation of the AYF mouse. (A) The targeted *Klra1* locus encoding Ly49A is depicted before and after removal of the neomycin resistance gene by Cre recombinase. LoxP sites are indicated as black triangles. The AYF allele contains a point mutation in exon 4 indicated as 4* that codes for Ly49A^{Y8F} with a tyrosine-to-phenylalanine mutation in the ITIM of Ly49A. (B) Parallel bars indicate the binding sites of probes that were used to generate the Southern blot on genomic DNA digested with PmeI and KpnI from ES cells that were homozygous for WT *Klra1* or heterozygous for the targeted allele. Bands representing the 3' and 5' arms are indicated. Removal of the neomycin resistance gene was confirmed by PCR.

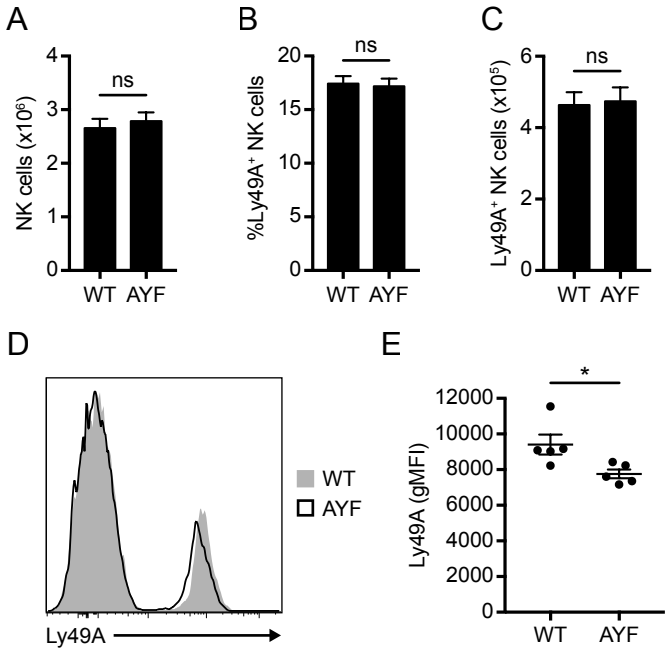


Figure 2.2: Ly49A expression on WT and AYF NK cells. (A) Total splenic NK cell ($CD3^-$ $CD19^-$ $NK1.1^+$ $NKp46^+$) numbers from WT and AYF mice. (B) Percentage of splenic NK cells that express Ly49A. (C) Absolute number of splenic $Ly49A^+$ NK cells. (D) Representative histogram showing Ly49A expression on NK cells. (E) Summary of the geometric MFI (gMFI) of Ly49A on $Ly49A^+$ NK cells. Data in (A-C) are pooled from 3 independent experiments with 13 mice per group total. Data in (D-E) is representative of 3 independent experiments with 13 mice per group total. * $p < 0.05$; ns = not significant (Student's *t*-test).

2.2.2 ITIM signaling is required for Ly49A to inhibit NK cell killing

To test if the ITIM is required for Ly49A to inhibit killing by primary murine NK cells, chromium release assays were performed with purified Ly49A$^+$ LAKs from WT B6 or homozygous AYF mice, in a manner similar to our original assays (Karlhofer et al., 1992). Ly49A$^+$ LAKs from WT and AYF mice exhibited similar levels of cytotoxicity towards C1498 (H-2^b) target cells at high effector-to-target (E:T) ratios (Fig. 2.3A). C1498-D12 target cells that express transfected H-2D^d were not killed by Ly49A$^+$ LAKs from WT mice due to the inhibitory interaction between Ly49A and H-2D^d as previously described (Karlhofer et al., 1992). In contrast, Ly49A$^+$ LAKs from AYF mice killed C1498-D12 targets at higher levels than WT LAKs, at all high E:T ratios tested (Fig. 2.3B). These data indicate that ITIM signaling is required for Ly49A to inhibit cytotoxicity by primary NK cells and that the AYF allele functionally inactivates the Ly49A ITIM.

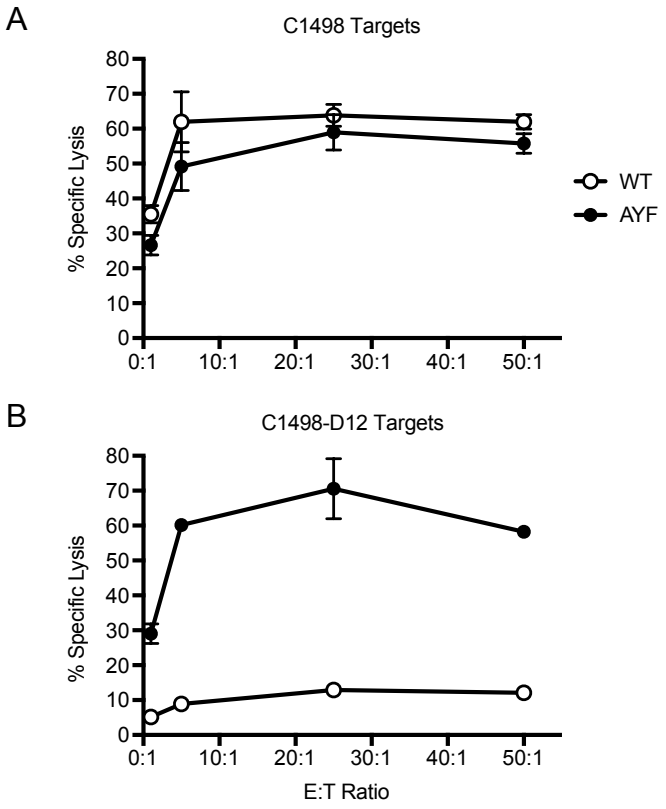


Figure 2.3: The ITIM is required for Ly49A to inhibit NK cell cytotoxicity. Chromium release assays were performed at the indicated effector-to-target (E:T) ratios using Ly49A^{+} LAKs from WT or AYF mice. (A) C1498 (H-2^{b}) or (B) C1498 transfected with H-2D^{d} (C1498-D12) cell lines were used as targets. LAKs were generated from splenocytes pooled from 3 mice per group. Error bars indicate SD of technical triplicates. Data are representative of 2 independent experiments.

2.2.3 Mutation of the Ly49A ITIM does not affect NK cell development

To study the function of Ly49A ITIM signaling *in vivo*, we bred the AYF mouse to KODO mice that lack expression of H-2K^b and H-2D^b (AYF KODO) and we introduced the ligand for Ly49A by breeding these mice to the D8 mouse that expresses an H-2D^d transgene to generate the AYF D8 KODO mouse. Although bone marrow competition experiments with Ly49A transgenic mice suggested that the Ly49A-H-2D^d interaction promotes NK cell development (Lowin-Kropf and Held, 2000), we found that total NK cell number and Ly49A$^+$ NK cell number were both unchanged between D8 KODO and AYF D8 KODO mice (Fig. 2.4A-C). Furthermore, mutation of the ITIM of Ly49A did not alter the maturation profile of Ly49A$^+$ NK cells as indicated by CD27 and CD11b expression (Fig. 2.4D-E). Thus, these data indicate that Ly49A ITIM signaling is not required for NK cell development.

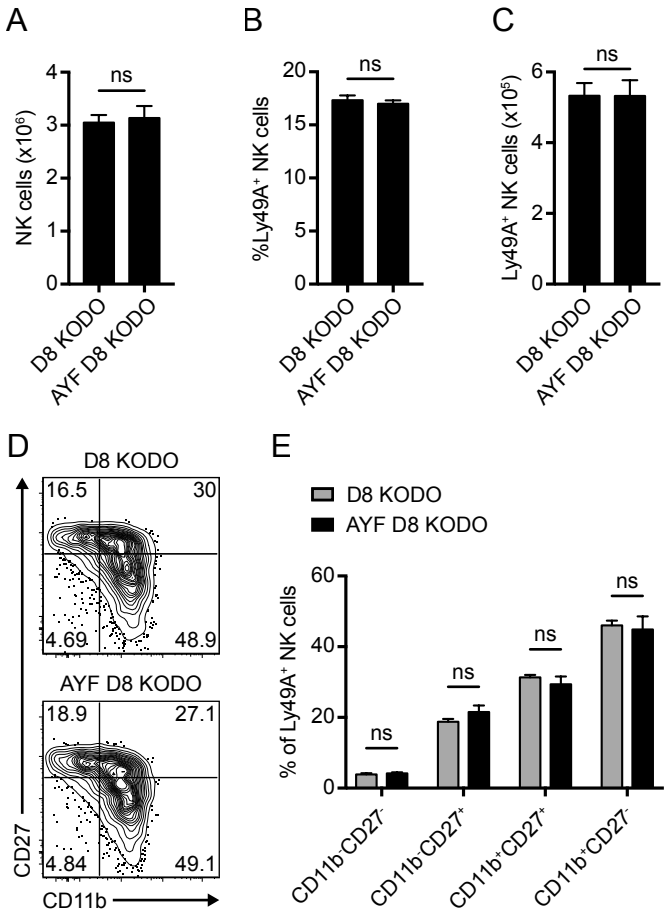


Figure 2.4: NK cell development is normal in AYF D8 KODO mice. (A) Total splenic NK cell ($CD3^-$ $CD19^-$ $NK1.1^+$ $NKp46^+$) number, (B) percentage of NK cells that express Ly49A, and (C) $Ly49A^+$ NK cell number from D8 KODO and AYF D8 KODO mice for 16-20 mice per group. Data are pooled from at least 5 independent experiments. There were no significant differences as indicated (Student's *t*-test). (D) Representative dot plots of $Ly49A^+$ NK cell maturation stages. (E) Maturation stages of $Ly49A^+$ NK cells separated by CD27 and CD11b expression. Data in (E) are pooled from 2 independent experiments with a total of 6 mice per group (Two way ANOVA with Bonferroni correction). Error bars indicate SEM. ns = not significant.

2.2.4 NK cell licensing by Ly49A is ITIM-dependent

To investigate the role of ITIM signaling in NK cell licensing, we stimulated NK cells from KODO, AYF KODO, D8 KODO, and AYF D8 KODO mice with plate-bound anti-NK1.1 antibody and analyzed IFN-γ production, as described previously (Fernandez et al., 2005; Kim et al., 2005). To quantify the degree of NK cell licensing by a given Ly49, a licensing ratio was calculated as the ratio of the percentage of IFN-γ^+ cells within the $Ly49^+$ subset over the $Ly49^-$ subset as previously described (Jonsson and Yokoyama, 2010). $Ly49G2^+$ and $NKG2A^+$ NK cells were gated out prior to calculating the licensing ratio for Ly49A in order to eliminate the potential confounding effects of licensing through these other receptors by $H\text{-}2D^d$. $Ly49A^+$ NK cells from the MHC-I-deficient KODO and AYF KODO mice produced little IFN-γ after stimulation with anti-NK1.1, which corresponded to a licensing ratio of less than 1 (Fig. 2.5A-B). In contrast, a large fraction of $Ly49A^+$ NK cells from D8 KODO mice produced IFN-γ due to licensing by Ly49A on $H\text{-}2D^d$, corresponding to a Ly49A licensing ratio of greater than 1 in D8 KODO mice, as previously reported (Choi et al., 2011; Kim et al., 2005). Remarkably, $Ly49A^+$ NK cells from AYF D8 KODO mice produced dramatically lower levels of IFN-γ compared to D8 KODO mice with a reduction in the Ly49A licensing ratio to below 1 in AYF D8 KODO mice (Fig. 2.5A-B). Importantly, when activation receptor signaling was bypassed with PMA and ionomycin stimulation, $Ly49A^+$ and $Ly49A^-$ NK cells from all strains produced equivalent levels of IFN-γ (Fig. 2.5C), verifying that $Ly49A^+$ NK cells from AYF D8 KODO mice remained capable of producing IFN-γ. Thus, licensing through the interaction between Ly49A and $H\text{-}2D^d$ is completely abolished in AYF D8 KODO mice.

To test whether licensing through other Ly49 receptors is affected by the AYF mutation, we assessed NK cell licensing by Ly49G2 in $Ly49A^-$ $NKG2A^-$ NK cells after stimulation with

plate-bound anti-NK1.1 antibody. The Ly49G2 licensing ratio was elevated in D8 KODO compared to KODO mice (Fig. 2.5D-E), indicating that Ly49G2^{+} NK cells were licensed by H-2D^{d} as has been previously suggested (Sun et al., 2012). In contrast to the Ly49A licensing ratio (Fig. 2.5A-B), the Ly49G2 licensing ratio was unchanged between D8 KODO and AYF D8 KODO mice (Fig. 2.5D-E). Ly49G2^{+} and Ly49G2^{-} NK cells from all strains responded equivalently to stimulation with PMA and ionomycin (Fig. 2.5F). Collectively, these data indicate that mutation of the ITIM of Ly49A impairs NK cell licensing in a manner that is cell-intrinsic to Ly49A-expressing cells.

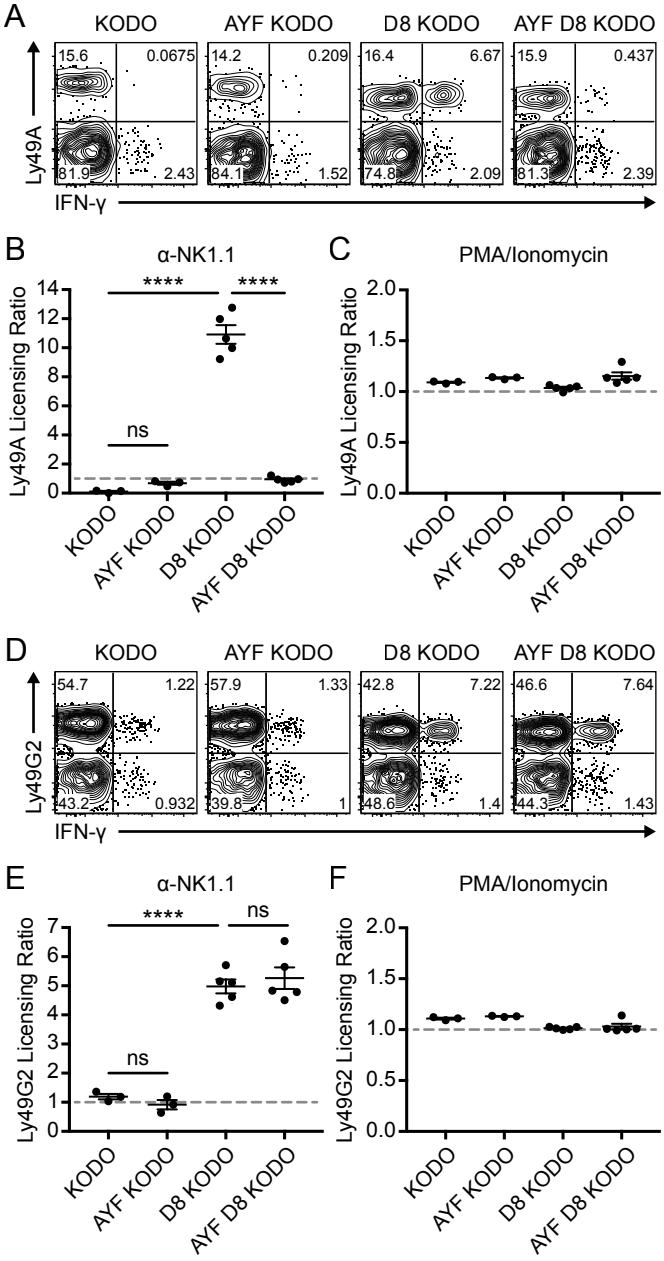


Figure 2.5: NK cell licensing by Ly49A is impaired in AYF D8 KODO mice. Splenocytes from KODO, AYF KODO, D8 KODO, and AYF D8 KODO mice were stimulated with plate-bound anti-NK1.1 antibody or with PMA and ionomycin and analyzed for intracellular IFN-γ production by NK cells ($CD3^-$ $CD19^-$ $NKp46^+$). (A) Representative dot plots showing IFN-γ production by $Ly49G2^-$ $NKG2A^-$ NK cells after stimulation with plate-bound anti-NK1.1 antibody. (B-C) Relative production of IFN-γ by $Ly49A^+$ and $Ly49A^-$ cells within the $Ly49G2^-$

NKG2A$^-$ NK cell population is quantified by a licensing ratio (see Materials and Methods) for stimulation with (B) plate-bound anti-NK1.1 or (C) PMA and ionomycin. (D) Representative dot plots showing IFN-γ production by Ly49A$^-$ NKG2A$^-$ NK cells after stimulation with plate-bound anti-NK1.1 antibody. (E-F) Relative production of IFN-γ by Ly49G2$^+$ and Ly49G2$^-$ cells within the Ly49A$^-$ NKG2A$^-$ NK cell population is quantified by a licensing ratio for stimulation with (E) plate-bound anti-NK1.1 or (F) PMA and ionomycin. Data are representative of 4 independent experiments with a total of 12-15 mice per group. Error bars indicate SEM. **** $p < 0.0001$; ns = not significant (One-way ANOVA with Bonferroni correction).

2.2.5 Downregulation of the Ly49A MFI by H-2D^d does not require ITIM signaling

To determine whether ITIM signaling is required for the H-2D^d-dependent MFI shift in Ly49A, we compared the MFI of Ly49A in KODO, AYF KODO, D8 KODO, and AYF D8 KODO mice (Fig. 2.6). The MFI of Ly49A was reduced in D8 KODO compared to KODO mice as previously reported (Choi et al., 2011; Johansson et al., 2005). However, the MFI of Ly49A was also significantly downregulated in AYF D8 KODO mice compared to AYF KODO mice and we did not observe a difference in the MFI of Ly49A in D8 KODO and AYF D8 KODO mice (Fig. 2.6). We observed a very small but reproducible reduction in the Ly49A MFI in AYF KODO compared to KODO mice. We also observed a similar small reduction in the Ly49A MFI on NK cells from AYF compared to WT mice (Fig. 2.2D-E), suggesting that ITIM signaling may modulate the expression level of Ly49A in the absence of classical MHC-I molecules expressed as self or that the Ly49A targeting strategy slightly altered Ly49A expression. Overall, these data show that H-2D^d downregulates the MFI of Ly49A in an ITIM-independent manner, which argues against a role for signaling-dependent internalization of Ly49A by classical MHC-I molecules expressed as self.

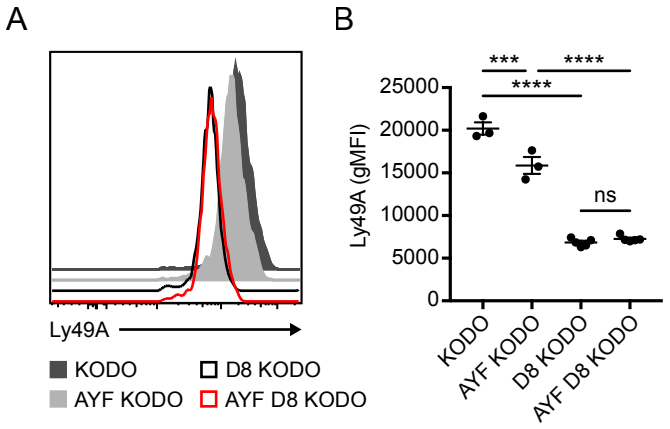


Figure 2.6: H-2D^{d}-dependent downregulation of the Ly49A MFI is ITIM-independent. (A) Representative histogram of Ly49A expression on Ly49A^{+} splenic NK cells (CD3^{-} CD19^{-} NK1.1^{+} NKp46^{+} Ly49A^{+}) from KODO, AYF KODO, D8 KODO, and AYF D8 KODO mice. (B) Summary of the geometric MFI (gMFI) of Ly49A on Ly49A^{+} NK cells from the indicated strains. Data are representative of 4 independent experiments with a total of 10-13 mice per group. Error bars indicate SEM. **** $p < 0.0001$; *** $p < 0.001$; ns = not significant (One-way ANOVA with Bonferroni correction).

2.2.6 Ly49A ITIM signaling regulates the NK cell receptor repertoire

To determine the role of ITIM signaling in the development of the NK cell receptor repertoire, we assessed the expression of Ly49s and NKG2A/CD94 in splenic NK cells freshly isolated from KODO, AYF KODO, D8 KODO, and AYF D8 KODO mice (Fig. 2.7). As previously reported in H-2^d mice (Held et al., 1996b), there was a substantial reduction in percentage of Ly49A$^+$ cells expressing Ly49G2 in D8 KODO when compared to KODO mice. Moreover, we observed a significant reduction in percentage of Ly49A$^+$ cells expressing Ly49F and Ly49I in D8 KODO mice as compared to KODO mice. Remarkably, there were substantially elevated percentages of Ly49A$^+$ NK cells expressing Ly49G2, Ly49F, and Ly49I in AYF D8 KODO compared to D8 KODO mice (Fig. 2.7). In contrast, we did not detect any significant differences in the percentages of NK cells expressing these Ly49s on Ly49A$^-$ NK cells from D8 KODO and AYF D8 KODO mice (Fig. 2.8B), which suggests that these repertoire changes are not due to off-target mutations in other *Ly49* genes. Thus, these data suggest that the effect of Ly49A ITIM signaling on the receptor repertoire is cell-intrinsic to Ly49A-expressing cells and that ITIM signaling is required for H-2D^d-dependent skewing of the receptor repertoire on Ly49A$^+$ NK cells.

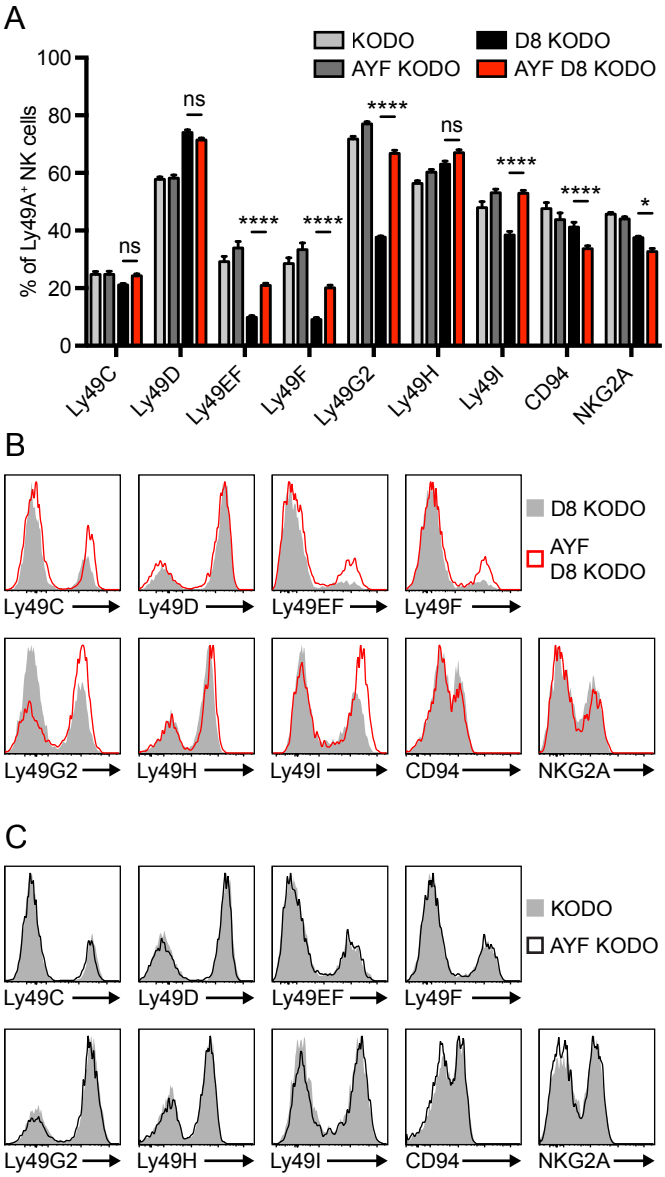


Figure 2.7: Mutation of the Ly49A ITIM causes skewing of the NK cell receptor repertoire. (A) Summary of receptor expression on splenic Ly49A$^+$ NK cells (CD3$^-$ CD19$^-$ NK1.1$^+$ NKp46$^+$ Ly49A$^+$). (B) Representative histograms showing receptor expression on Ly49A$^+$ NK cells from D8 KODO and AYF D8 KODO mice. (C) Representative histograms showing receptor expression on Ly49A$^+$ NK cells from KODO and AYF KODO mice. Data is pooled from 3 independent experiments with 8-11 mice per group total. **** $p < 0.0001$; * $p < 0.05$ (Two-way ANOVA with Bonferroni correction).

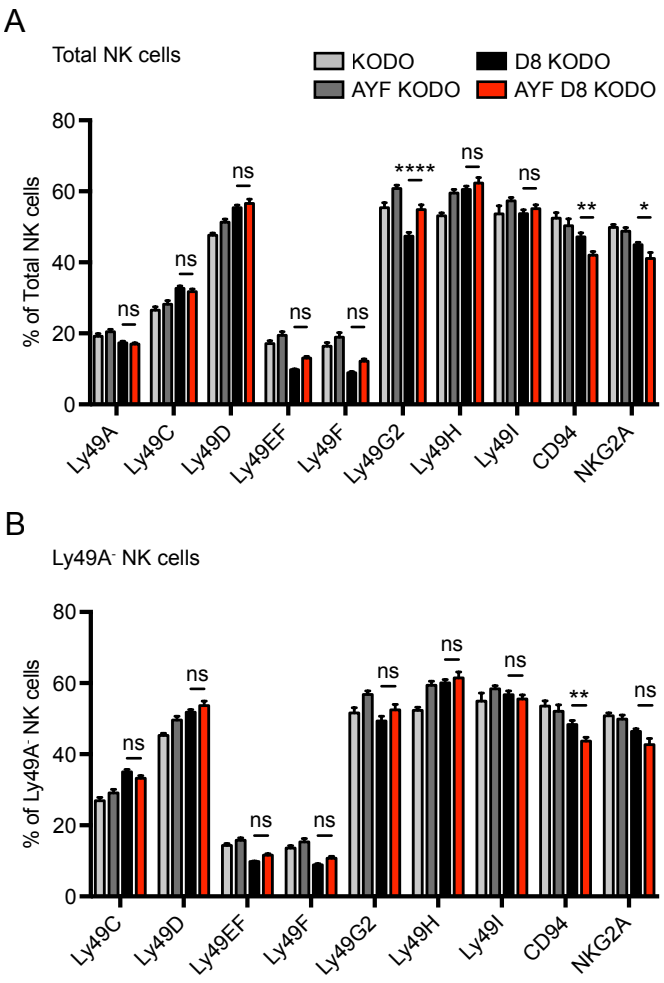


Figure 2.8: Receptor repertoire on total and Ly49A$^-$ NK cells. Summary of receptor expression on (A) splenic NK cells (CD3$^-$ CD19$^-$ NK1.1$^+$ NKp46$^+$) and (B) Ly49A$^-$ NK cells. Data is pooled from at least 3 independent experiments with 8-16 mice per group total. **** $p <$ 0.0001; ** $p < 0.01$; * $p < 0.05$; ns = not significant (Two-way ANOVA with Bonferroni correction).

2.3 Discussion

Here we describe the first knock-in mouse with a targeted mutation in a self-MHC-specific Ly49 expressed by mature NK cells. The AYF mouse expresses Ly49A with a point mutation known to inactivate ITIM signaling *in vitro*, and here we show that Ly49A ITIM signaling inhibits cytotoxicity by primary NK cells. This mouse allowed us to definitively study the role of the ITIM in the functions and attributes of a self-MHC-specific inhibitory NK cell receptor. To study the role of Ly49A in NK cell development and education, we bred AYF D8 KODO mice that express the ligand for Ly49A, H-2D^{d}, and lack all other classical MHC-I molecules. We used AYF D8 KODO mice to show that ITIM signaling is not required for NK cell development but is required for NK cell licensing. Moreover, we show that ITIM signaling is not required for H-2D^{d} to downregulate the Ly49A MFI. In contrast, our data provide evidence that Ly49A ITIM signaling significantly shapes the NK cell receptor repertoire.

2.4 Materials and Methods

2.4.1 Mice

C57BL/6 (B6) mice were purchased from the National Cancer Institute (Frederick, MD) and from Charles River Laboratories (Wilmington, MA). Albino B6 mice (B6(Cg)-*Tyr*$^{c-2J}$/J) were purchased from The Jackson Laboratory. H-2K$^{b-/-}$ H-2D$^{b-/-}$ double knockout (KODO) mice were purchased from Taconic Farms. D8 transgenic mice expressing an H-2D^{d}-transgene were provided by D. Marguiles (National Institute of Allergy and Infectious Diseases, Bethesda, MD). D8 KODO mice were generated by crossing D8-transgenic mice to KODO mice. CMV-Cre mice (Schwenk et al., 1995) backcrossed to the C57BL/6 background were provided by Marco Colonna (Washington University, St. Louis, MO).

2.4.2 Development of AYF mice

BAC recombineering (Red/ET, GeneBridges) was used to subclone an 11.9 kb region of *Klra1* from BAC RP23-44607 (ACO87336, Children's Hospital Oakland Research Institute) into the pACYC177 vector (New England Biolabs). The pACYC177 capture vector contained a 146 bp 5' capture arm and a 301 bp 3' capture arm. The loxP-pGK-gb2-neo-loxP selection cassette (GeneBridges) was cloned into the NsiI site between exons 4 and 5 of the *Klra1* gene. The tyrosine at position 8 of Ly49A was replaced with phenylalanine by cloning in a 122 bp PCR product with XhoI and Bsu36I. Primers used to generate the Ly49A^{Y8F} mutation were the following: 5'-CACTCGAGGCACCATTTGAACTGAGAACATACTTTATATATCAATCCCAAGATGAGTGAGCAGGAGGTCACTTTTTC-3', 5'-TTTAGTCTCCTCAGGTCTCACTTGTTTCTGCAATCCTGCAGATTTATGAAATCTCACCATTGAAAAAG-3'. The targeting construct was electroporated into C57BL/6NTac-derived embryonic stem (ES) cells generated in-house (Gan et al., 2014), and positive clones were screened by Southern blot analysis. 7/370 ES cell clones were positive by Southern blot. Karyotyping revealed that 2/7 positive clones contained a normal karyotype. Positive clones with a normal karyotype were microinjected into Albino B6 blastocysts by the Transgenic, Knockout, and Micro-Injection Core at Washington University. Chimeric mice were bred to Albino B6 mice, and the targeted allele from one of the two ES cell lines was germline-transmitted. Mice containing the germline-transmitted targeted *Klra1* allele were bred to C57BL/6 mice to remove the Albino allele and subsequently to C57BL/6 mice that express a Cre transgene under the control of the CMV promoter (CMV-Cre) (Schwenk et al., 1995) to delete the selection cassette. The CMV-Cre transgene was removed by breeding to C57BL/6. The AYF allele was then bred to homozygosity for all experiments. AYF mice were subsequently crossed to KODO or D8

KODO mice to generate the AYF KODO and AYF D8 KODO strains respectively. D8 KODO and AYF D8 KODO mice were homozygous for the D8 transgene in all experiments.

2.4.3 Antibodies and Flow Cytometry

The following antibodies and reagents were purchased from eBioscience: anti-CD3e (145-2C11), anti-CD19 (eBio1D3), anti-NK1.1 (PK136), anti-NKp46 (29A1.4), anti-CD27 (LG.7F9), anti-CD11b (M1/70), anti-Ly49D (eBio4E5), anti-Ly49E/F (CM4), anti-Ly49G2 (eBio4D11), anti-Ly49H (3D10), anti-Ly49I (YLI-90), anti-CD94 (18d3), anti-NKG2A^{B6} (16a11), anti-IFNγ (XMG1.2), Fixable Viability Dye eFluor 506. The following antibodies and reagents were purchased from BD Biosciences: anti-Ly49F (HBF-719), anti-Ly49G2 (4D11), streptavidin PE (SA-PE). The following antibodies and reagents were purchased from BioLegend: anti-NK1.1 (PK136), streptavidin APC (SA-APC). Anti-Ly49I (YLI-90) was purchased from Abcam. Anti-Ly49A (JR9) was purified in our laboratory from hybridoma supernatants and subsequently conjugated to biotin or FITC. The JR9 hybridoma was generously provided by Jacques Roland (Pasteur Institute, Paris, France). Anti-Ly49C (4LO33) was purified in our laboratory from hybridoma supernatants and subsequently conjugated to biotin. The 4LO hybridoma was generously provided by Suzanne Lemieux (Institut National de la Recherche Scientifique-Institut Armand-Frappier, Laval, Quebec, Canada). Anti-NK1.1 (PK136) was purified in our laboratory from hybridoma supernatants. The PK136 hybridoma was purchased from American Type Culture Collection (Manassas, VA). Fc receptor blocking was performed with 2.4G2 (anti-FcγRII/III) hybridoma (American Type Culture Collection, Manassas, VA) culture supernatants. Surface staining was performed on ice in staining buffer (1% BSA, 0.01% NaN_3 in PBS). Samples were collected using a FACSCanto (BD Biosceinces), and data were analyzed using FlowJo (Tree Star, Ashland, OR).

2.4.4 Preparation of Ly49A$^+$ lymphokine-activated killer cells (LAKs)

Nylon-wool non-adherent splenocytes were cultured in R10 media supplemented with 800 IU/mL of IL-2. On day 6, Ly49A$^+$ LAKs were purified by panning with the JR9 (anti-Ly49A) antibody as previously described (Choi et al., 2011; Karlhofer et al., 1992). LAKs were harvested for chromium release assays on day 9 or 10. LAKs were 94-97% Ly49A$^+$ by flow cytometry.

2.4.5 Chromium release assay

Four-hour ^{51}Cr release assays were performed as previously described (Karlhofer et al., 1992). Day 9 or 10 Ly49A$^+$ LAKs were used as effectors. C1498 target cells were purchased from American Type Culture Collection (Manassas, VA). The C1498-D12 target cell line was generated previously by transfecting C1498 cells with H-2D^d (Karlhofer et al., 1992).

2.4.6 *In vitro* stimulation and intracellular cytokine staining

Splenocytes were stimulated with anti-NK1.1 (PK136) as previously described (Jonsson and Yokoyama, 2010; Kim et al., 2005). Briefly, 24-well culture plates were coated with 500µl of purified PK136 (1µg/mL). Plates were washed with PBS, and then 5x10^6 splenocytes were added to each well in 500µl of R10 media. Splenocytes were stimulated in parallel with 0.5µg/mL PMA (Sigma-Aldrich) and 4µg/mL ionomycin (Sigma-Aldrich). Splenocytes were incubated at 37°C and 5% CO_2 for a total of 7 hours. Brefeldin A (GolgiPlug, BD Biosciences) was added to the cells after 1 hour. After staining surface antigens, cells were fixed and permeabilized (Cytofix/Cytoperm; BD Biosciences) followed staining for IFN-γ. NK cells were gated as Viable CD3$^-$ CD19$^-$ NKp46$^+$ lymphocytes.

The licensing ratio was calculated as [(%Ly49$^+$IFN-γ$^{+)}$/(%Ly49$^+$)]/[(%Ly49$^-$IFN-γ$^+$)/(%Ly49$^-$)] as previously described (Jonsson and Yokoyama, 2010). The Ly49A licensing

ratio was calculated on Ly49G2$^-$ NKG2A$^-$ NK cells, and the Ly49G2 licensing ratio was calculated on Ly49A$^-$ NKG2A$^-$ NK cells.

Chapter 3: Inflammation drives natural killer cell missing-self reactivity

Published in the *Journal of Experimental Medicine* (Bern et al., 2019).

3.1 Introduction

B2m$^{-/-}$ mice do not exhibit NK cell-mediated autoimmunity in contrast to the predictions of the missing-self hypothesis (Kärre et al., 1986). Our lab previously proposed that NK cells from *B2m*$^{-/-}$ mice are self-tolerant because they are uneducated (Kim et al., 2005). This hypothesis predicts that acute downregulation of MHC-I in a mouse that contains educated NK cells should induce NK cell autoreactivity. More recent studies, however, suggest that NK cells become re-educated in response to changes in the MHC-I environment to maintain self-tolerance (Elliott et al., 2010; Joncker et al., 2010). As a result, it is unclear if acute downregulation of MHC-I induces missing-self reactivity *in vivo* as proposed by the missing-self hypothesis.

Here, we generated mice in which *B2m* could be inducibly deleted to directly study the response of NK cells that developed in an MHC-I-sufficient setting to acute downregulation of MHC-I *in vivo*. MHC-I downregulation was found to induce multiple NK cell responses besides missing-self reactivity that depended on the context. Importantly, the inflammatory environment in which MHC-I was downregulated was found to regulate NK cell missing-self reactivity.

3.2 Results

3.2.1 Generation of conditional *B2m* knockout mice

To develop a mouse in which MHC-I expression could be downregulated, we generated floxed *B2m* mice. C57BL/6 embryonic stem (ES) cells were obtained with a *B2m* knockout first allele with conditional potential (*B2m*^{tm1a}) (Fig. 3.1A). The HEPD0673_4_D09 ES cell clone was

verified to be correctly targeted at the *B2m* locus by Southern blot analysis (Fig. 3.2) and was microinjected into Albino B6 blastocysts. Mice containing the germline-transmitted $B2m^{tm1a}$ allele were subsequently bred to a FLP transgenic line to generate the floxed *B2m* allele ($B2m^{fl}$).

To validate the $B2m^{fl}$ allele, floxed *B2m* mice were bred with CMV-Cre transgenic mice for ubiquitous expression of Cre to induce germline deletion of *B2m* ($B2m^{\Delta}$) (Fig. 3.1A). Splenocytes from B6 (WT) and $B2m^{fl/fl}$ mice both expressed surface H-2K^b and H-2D^b (Fig. 3.1B). In contrast, the original germline *B2m* knockout ($B2m^{-/-}$) and the $B2m^{\Delta/\Delta}$ mouse both lacked surface expression of MHC-I (Fig. 3.1B). In addition, $B2m^{\Delta/\Delta}$ mice were deficient in $CD8^+$ T cells (Fig. 3.1C-D), consistent with impaired positive selection of cytotoxic T cells as originally described in $B2m^{-/-}$ mice (Koller et al., 1990; Zijlstra et al., 1990). Furthermore, splenocytes from $B2m^{\Delta/\Delta}$ mice were rejected after transfer into WT mice to a similar extent as $B2m^{-/-}$ splenocytes, and this rejection was abrogated by depletion of NK cells with the anti-NK1.1 antibody (Fig. 3.1E-F), as previously described (Oberg et al., 2004). Taken together, these data indicate that $B2m^{fl/fl}$ mice express MHC-I and that Cre mediates *B2m* deletion resulting in cells from $B2m^{\Delta/\Delta}$ mice that lack surface MHC-I and are targets for missing-self recognition.

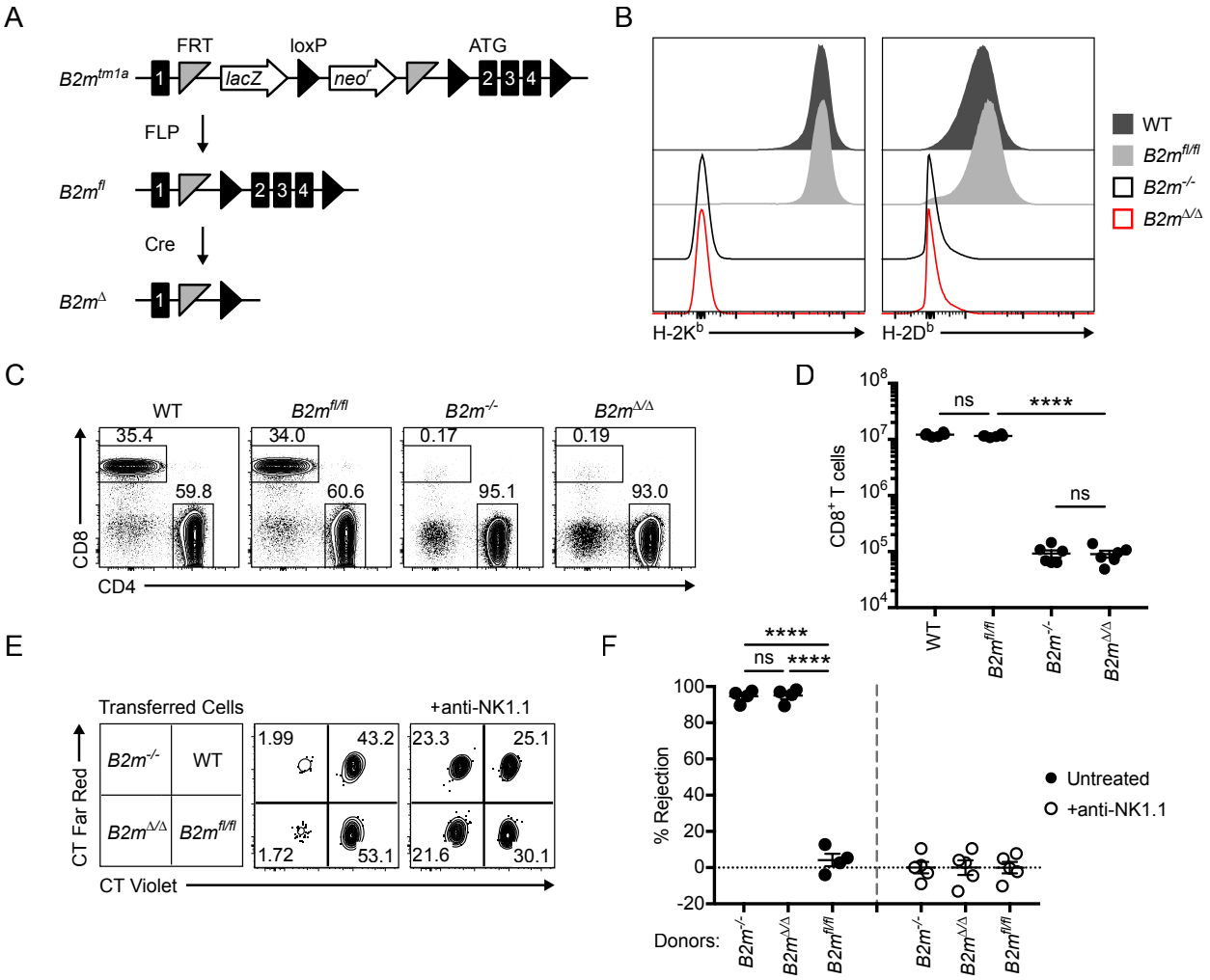


Figure 3.1: Conditional deletion of *B2m* leads to loss of surface MHC-I. (A) The targeted *B2m*^{tm1a} allele is depicted before (top) and after (middle) removal of the *LacZ* and neomycin resistance (*neo*^{r}) genes by FLP recombinase. Germline-expressed Cre recombinase was used to generate the *B2m*$^{\Delta}$ allele (bottom). (B) Representative histograms of H-2K^{b} and H-2D^{b} expression on total lymphocytes from spleens of WT, *B2m*$^{fl/fl}$, *B2m*$^{-/-}$, and *B2m*$^{\Delta/\Delta}$ mice. (C) Representative dot plots showing the percentage of T cells (CD19^{-} CD3^{+}) that express CD4 or CD8. Data in (B) and (C) are representative of 2 independent experiments with 3 mice per group. (D) Total splenic CD8^{+} T cell number in WT, *B2m*$^{fl/fl}$, *B2m*$^{-/-}$, and *B2m*$^{\Delta/\Delta}$ mice (n = 6 mice per

group). Data in (D) are combined from 2 independent experiments. (E-F) Splenocytes from WT, *B2m*$^{fl/fl}$, *B2m*$^{-/-}$, and *B2m*$^{\Delta/\Delta}$ mice were labeled with CFSE and differentially labeled with CellTrace Violet (CT Violet) and CellTrace Far Red (CT Far Red) as indicated. Labeled cells were injected IV into WT recipient mice, and donor cells were recovered from spleens of recipients after 2 days. (E) Representative dot plots showing the relative percentages of transferred cells (CFSE^{+}) recovered from the spleens of WT recipient mice that were depleted of NK cells with anti-NK1.1 (right) or undepleted (middle). (F) Percent NK cell-specific rejection of donor cells by WT recipient mice (n = 4-5 mice per group). Data in (F) are representative of 2 independent experiments with 4-5 mice per group per experiment. Statistical significance was calculated by one-way ANOVA. Error bars indicate mean ± SEM.

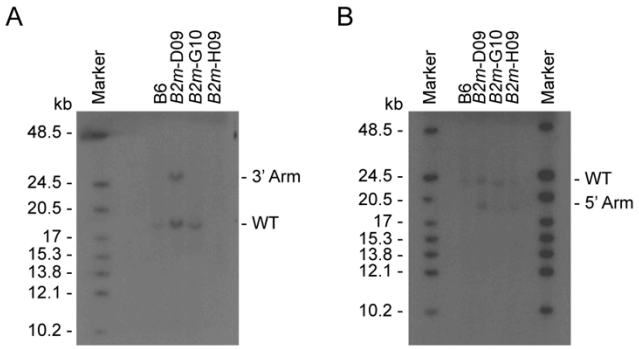


Figure 3.2: Southern blot analysis of *B2m*^{tm1a} ES cells. Southern blot on genomic DNA from B6 ES cells and three different *B2m*^{tm1a} ES cell clones: HEPD0673_4_D09 (*B2m*-D09), HEPD0673_4_G10 (*B2m*-G10), and HEPD0673_4_H09 (*B2m*-H09). Genomic DNA from ES cells was digested with (A) AhdI to probe for the 3' homology arm or (B) PmeI and EcoRV to probe for the 5' homology arm. The *B2m*-D09 ES cell clone was found to be correctly targeted at the *B2m* locus. The *B2m*-D09 ES cell clone was used to generate the *B2m*^{tm1a} mouse.

3.2.2 NK cell missing-self reactivity is not observed after global downregulation of MHC-I

To study the *in vivo* NK cell response to acute downregulation of MHC-I, floxed *B2m* mice were bred to Rosa26-Cre-ER^{T2} (R26-Cre-ER^{T2}) mice that express a tamoxifen-inducible Cre in all cells. We expected that tamoxifen treatment of *B2m$^{fl/fl}$* R26-Cre-ER^{T2} mice would induce the accumulation of MHC-I-deficient cells only when NK cells were depleted. Surprisingly, tamoxifen induced the accumulation of a substantial population of MHC-I-deficient cells in *B2m$^{fl/fl}$* R26-Cre-ER^{T2} mice even in the presence of NK cells (Fig. 3.3A-B). In addition, depletion of NK cells with the anti-NK1.1 antibody did not lead to any observed increase in MHC-I-deficient cells (Fig. 3.3A-B). These data suggest that global downregulation of MHC-I does not induce overt NK cell missing-self reactivity as predicted by the missing-self hypothesis (Kärre et al., 1986).

To investigate the reasons why NK cell reactivity was not observed after global downregulation of MHC-I, we first used conventional assays to test whether cells from *B2m$^{fl/fl}$* R26-Cre-ER^{T2} mice fail to become targets for missing-self recognition by NK cells after tamoxifen-treatment (Fig. 3.3C-D). *B2m$^{fl/fl}$*, *B2m$^{fl/fl}$* R26-Cre-ER^{T2}, and *B2m$^{\Delta/\Delta}$* mice were treated with either tamoxifen or vehicle control (corn oil), and splenocytes from these mice were subsequently transferred into WT recipients. NK cell-specific rejection was quantified by calculating the relative recovery of donor cell populations normalized to NK cell-depleted recipient mice. Remarkably, splenocytes from *B2m$^{fl/fl}$* R26-Cre-ER^{T2} mice were rejected by NK cells only when the donor mice were treated with tamoxifen (Fig. 3.3C-D). Control *B2m$^{fl/fl}$* splenocytes were not rejected when donor mice received tamoxifen. In contrast, *B2m$^{\Delta/\Delta}$* splenocytes were strongly rejected by NK cells regardless of whether the donor mice received tamoxifen or corn oil. These data show that the MHC-I-deficient cells induced by tamoxifen in

B2m$^{fl/fl}$ R26-Cre-ER^{T2} mice become targets for missing-self recognition by NK cells upon adoptive transfer. However, NK cells in tamoxifen-treated *B2m*$^{fl/fl}$ R26-Cre-ER^{T2} mice appear to remain tolerant to these potential missing-self targets.

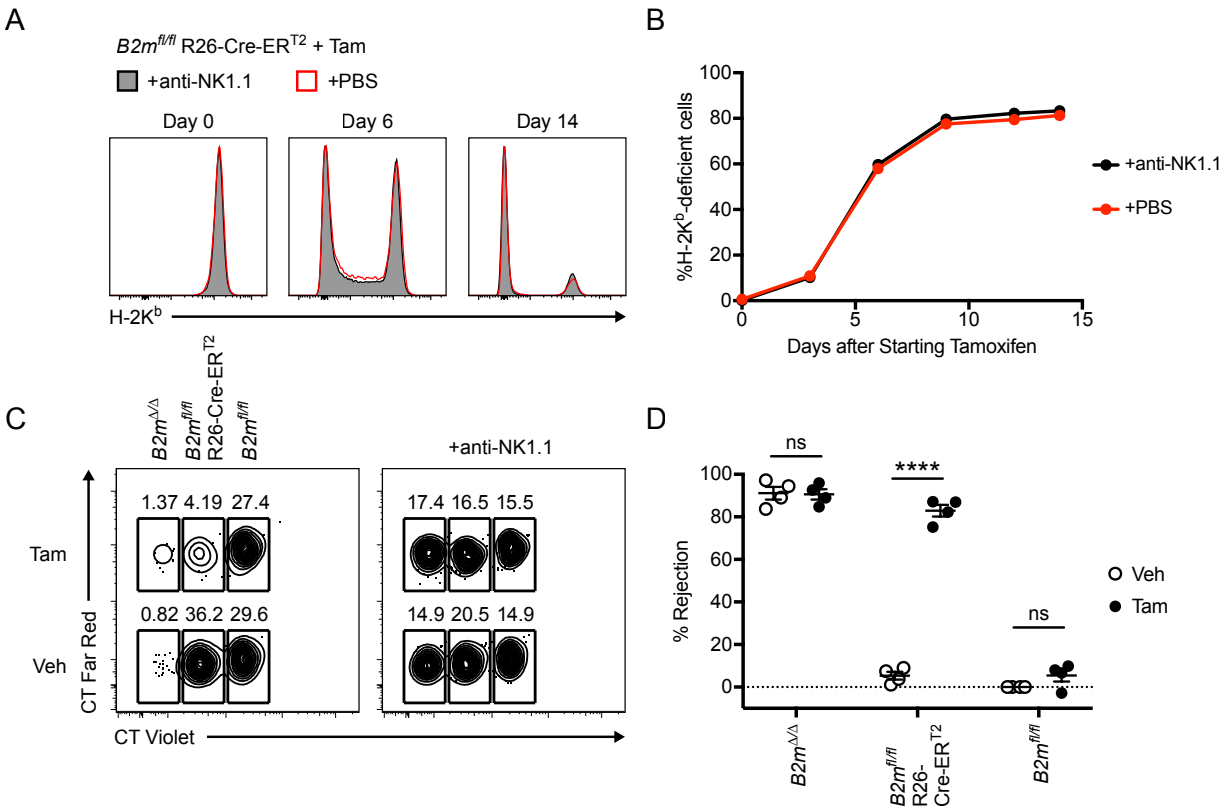


Figure 3.3: Global downregulation of MHC-I does not induce overt NK cell missing-self reactivity. (A) Representative histograms showing H-2K^b expression on CD45$^+$ lymphocytes from peripheral blood of *B2m*$^{fl/fl}$ R26-Cre-ER^{T2} mice treated with tamoxifen starting on day 0. Mice were injected IP with anti-NK1.1 antibody to deplete NK cells or with PBS control as indicated. (B) The percentage of H-2K^b-deficient CD45$^+$ cells that accumulate in the blood of tamoxifen-treated *B2m*$^{fl/fl}$ R26-Cre-ER^{T2} mice over time (n = 4 mice per group). Similar results to those in (A) and (B) were observed in a second experiment with 3 mice per group. (C-D) *B2m*$^{fl/fl}$, *B2m*$^{fl/fl}$ R26-Cre-ER^{T2}, and *B2m*$^{Δ/Δ}$ mice were treated with tamoxifen (Tam) or vehicle control (Veh) on days 0 through 4, and splenocytes were harvested and labeled with CFSE plus CT Violet and CT Far Red as indicated on day 16. Labeled splenocytes were IV injected into

WT recipients, and recovery of donor cells was analyzed after 2 days. (C) Representative dot plots showing the relative percentages of transferred cells ($CFSE^+$) recovered from the spleens of WT recipient mice that were depleted of NK cells with anti-NK1.1 (right) or undepleted (left). (D) NK cell-specific rejection of the indicated donor cells (n = 4 recipient mice; two-way ANOVA). Results in (C) and (D) are representative of 3 independent experiments in which donor cells were transferred on either day 14 or 16. Each symbol in (D) represents an individual recipient mouse from one replicate. CT Far Red labeling was reversed in one of 3 replicates. Error bars indicate mean ± SEM.

3.2.3 NK cells adapt to global downregulation of MHC-I

A previous attempt to generate a mouse with inducible deletion of an H-2D^d transgene driven by Mx1-Cre or CMV-Cre-ER was confounded by baseline leakiness of Cre expression that induced NK cell tolerance to H-2D^d-deficient cells in the absence of tamoxifen (Ioannidis et al., 2001). To test if there was similar leakiness of Cre in our *B2m$^{fl/fl}$* R26-Cre-ER^{T2} mice, we performed an *in vivo* cytotoxicity assay in *B2m$^{fl/fl}$* and *B2m$^{fl/fl}$* R26-Cre-ER^{T2} mice that had been pre-treated with vehicle control or tamoxifen (Fig. 3.4A). Remarkably, NK cells from *B2m$^{fl/fl}$* R26-Cre-ER^{T2} mice treated with vehicle control robustly rejected *B2m$^{\Delta/\Delta}$* splenocytes, although to a slightly lower extent than NK cells from *B2m$^{fl/fl}$* mice (Fig. 3.4B-C). In contrast, NK cells from tamoxifen-treated *B2m$^{fl/fl}$* R26-Cre-ER^{T2} mice were unable to reject *B2m$^{\Delta/\Delta}$* splenocytes (Fig. 3.4B-C). These results demonstrate that NK cells from *B2m$^{fl/fl}$* R26-Cre-ER^{T2} mice are initially capable of rejecting MHC-I-deficient cells; however, induced global MHC-I downregulation results in NK cell tolerance to missing-self.

Importantly, we did not observe any changes in NK cell number after global downregulation of MHC-I in *B2m$^{fl/fl}$* R26-Cre-ER^{T2} mice (Fig. 3.5A), and we observed only minor changes in NK cell maturation based on CD11b, CD27, and KLRG1 expression (Fig. 3.5B-C). Additionally the NK cell inhibitory receptor repertoire was relatively unchanged by global downregulation of MHC-I besides subtle changes in expression of Ly49C, Ly49F, and Ly49G2 (Fig. 3.5D). Thus, it seems unlikely that global downregulation of MHC-I induces NK cell tolerance through impacting NK cell development or the receptor repertoire.

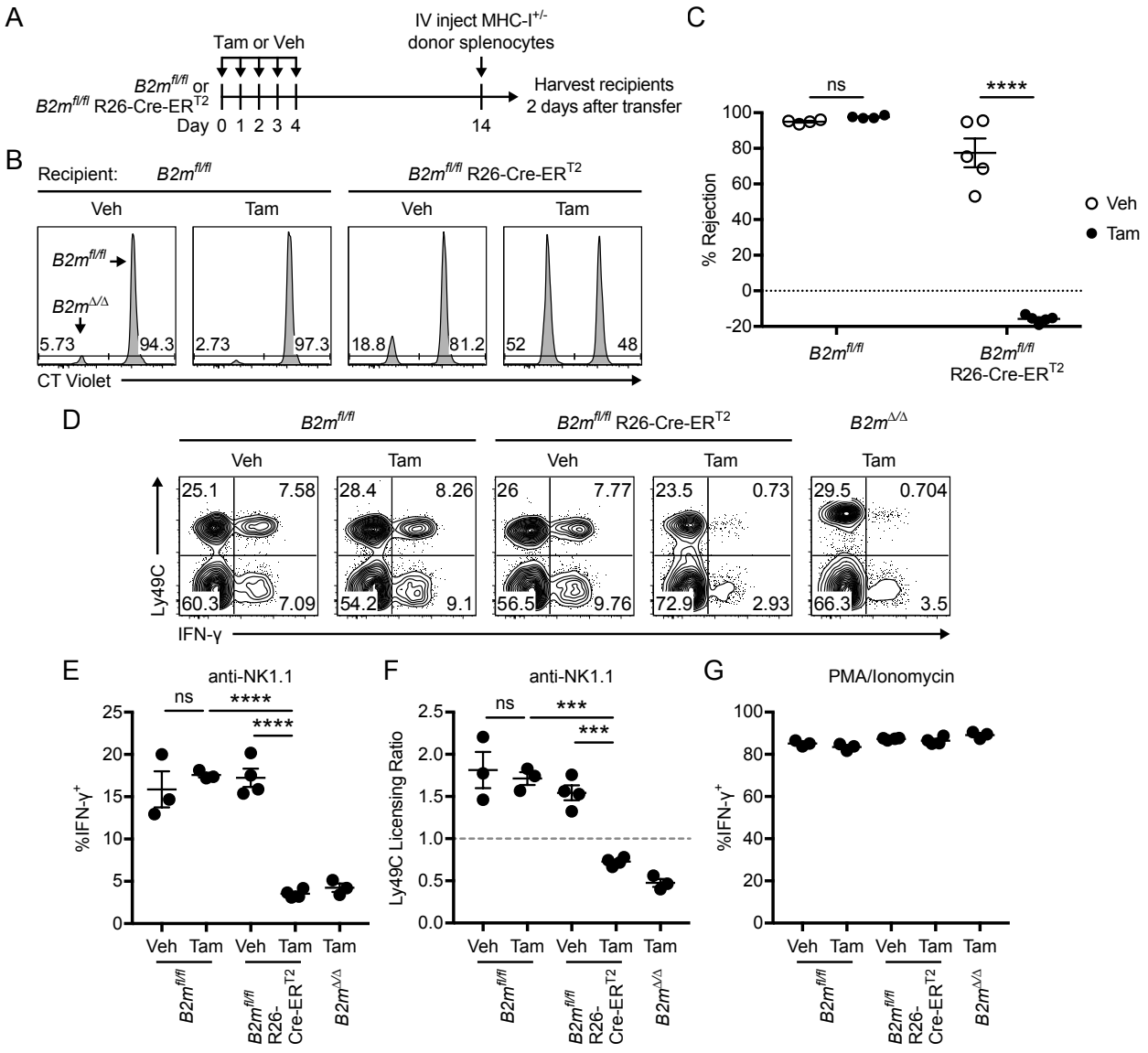


Figure 3.4: Global downregulation of MHC-I induces loss of NK cell licensing. (A) *B2m*$^{fl/fl}$ and *B2m*$^{fl/fl}$ R26-Cre-ER^{T2} mice were treated with tamoxifen (Tam) or vehicle control (Veh) on days 0 through 4 followed by IV injection of labeled donor splenocytes, as indicated, on day 14. Recipient spleens were harvested after 2 days to analyze donor cell recovery. (B) Representative histograms showing the relative percentages of transferred *B2m*$^{fl/fl}$ and *B2m*$^{\Delta/\Delta}$ cells (CFSE^{+} CT Far Red^{low}) recovered from the spleens of recipient mice. *B2m*$^{fl/fl}$ and *B2m*$^{\Delta/\Delta}$ cells were

differentially labeled with CT Violet as indicated. (C) Summary of NK cell-specific rejection of *B2m*$^{\Delta/\Delta}$ donor cells (n = 4-5 mice per group; two-way ANOVA). Data in (C) are combined from 2 independent experiments. Similar results were seen with WT and *B2m*$^{-/-}$ donor splenocytes (CFSE^{+} CT Far Red^{high}) that were co-transferred in these experiments. (D-G) *B2m*$^{fl/fl}$, *B2m*$^{fl/fl}$ R26-Cre-ER^{T2}, and *B2m*$^{\Delta/\Delta}$ mice were treated with tamoxifen or vehicle control on days 0 through 4, and splenocytes were stimulated on day 14 with plate-bound anti-NK1.1 antibody or PMA and ionomycin. (D) Representative dot plots showing percentage of NK cells (CD3^{-} CD19^{-} NKp46^{+}) that express Ly49C and IFN-γ after stimulation with plate-bound anti-NK1.1. (E) Percentage of NK cells that express IFN-γ after stimulation with plate-bound anti-NK1.1 (n = 3-4 mice per group; one-way ANOVA). (F) Relative production of IFN-γ by Ly49C^{+} and Ly49C^{-} NK cells after stimulation with anti-NK1.1 is quantified by a Ly49C licensing ratio (See Materials and Methods) (n = 3-4 mice per group; one-way ANOVA). (G) Percentage of NK cells that express IFN-γ after stimulation with PMA and ionomycin (n = 3-4 mice per group; one-way ANOVA). Data in (D-G) are representative of 2 independent experiments with 3-4 mice per group. Error bars indicate mean ± SEM.

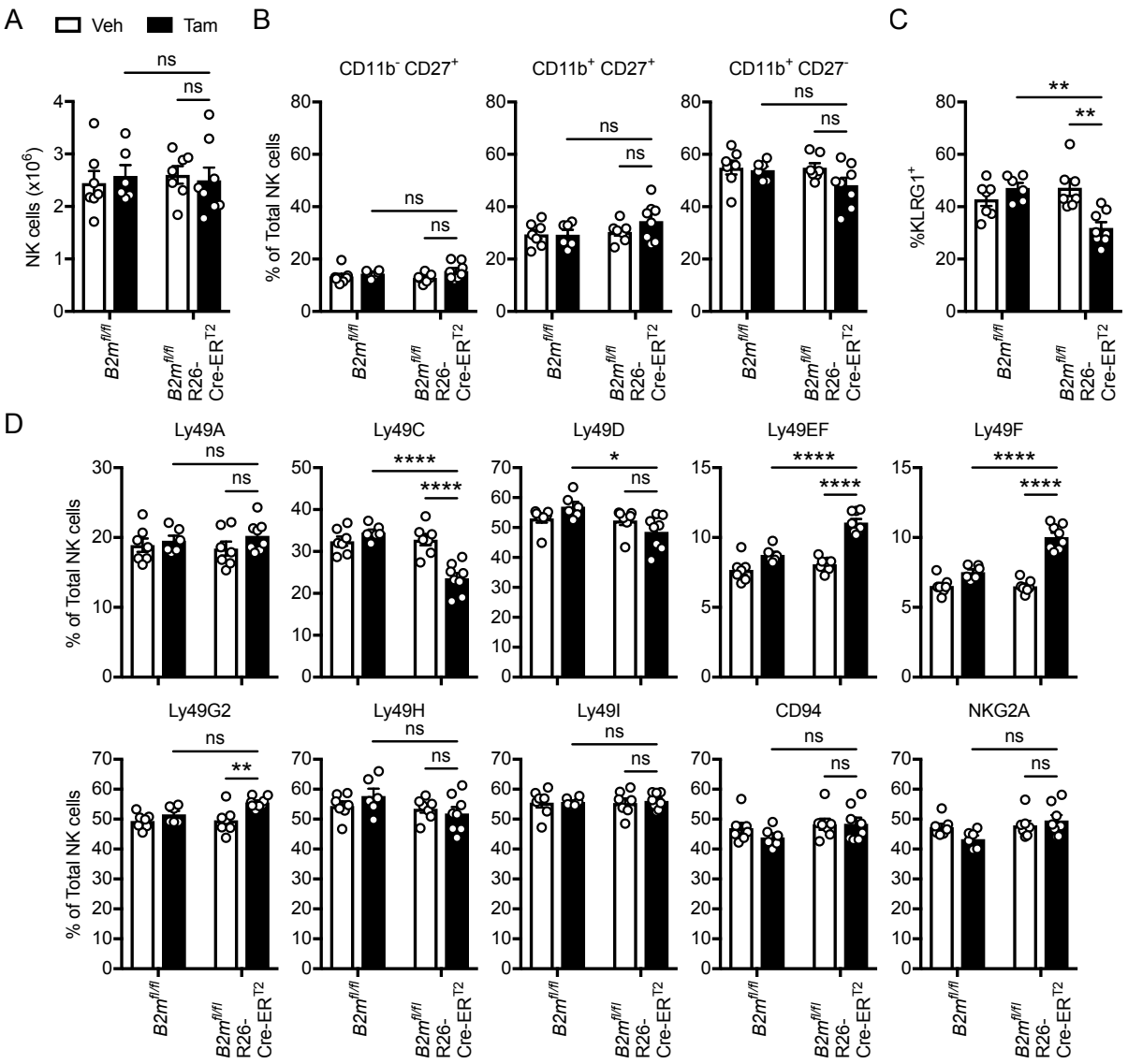


Figure 3.5: Global downregulation of MHC-I induces minor changes in NK cell surface phenotype. *B2m*$^{fl/fl}$ and *B2m*$^{fl/fl}$ R26-Cre-ER^{T2} mice were treated with tamoxifen (Tam) or vehicle control (Veh). (A) Number of splenic NK cells (CD3$^-$ CD19$^-$ NK1.1$^+$ NKp46$^+$) (n = 6-8 mice per group). (B) Percent of NK cells in different stages of NK cell maturation separated by expression of CD11b and CD27 (n = 6-8 mice per group). (C) Percent of NK cells that express the maturation marker KLRG1 (n = 6-8 mice per group). (D) Percent of NK cells that express the

indicated surface receptor (n = 6-8 mice per group). Data are combined from 2 independent experiments. Statistical significance was calculated by two-way ANOVA. Error bars indicate mean ± SEM.

Previous adoptive transfer studies have shown that WT NK cells can reset their responsiveness to stimulation through activation receptors when placed in an MHC-I-deficient environment (Joncker et al., 2010). To test if NK cells adapt to global loss of MHC-I, we treated *B2m*$^{fl/fl}$ and *B2m*$^{fl/fl}$ R26-Cre-ER^{T2} mice with tamoxifen or vehicle control and subsequently stimulated splenocytes with plate-bound anti-NK1.1 antibody as previously described (Jonsson and Yokoyama, 2010; Kim et al., 2005). IFN-γ production was analyzed as a measure of NK cell responsiveness, and a Ly49C licensing ratio was calculated to measure the preferential capacity of Ly49C^{+} NK cells to respond to stimulation (See Materials and Methods). As expected, only a small percentage of NK cells from *B2m*$^{\Delta/\Delta}$ mice produced IFN-γ after stimulation (Fig. 3.4D-E), which corresponded to a Ly49C licensing ratio below 1 (Fig. 3.4D, F). By contrast, a large fraction of control NK cells from *B2m*$^{fl/fl}$ mice produced IFN-γ after anti-NK1.1 stimulation regardless of whether or not the mouse was pre-treated with tamoxifen (Fig. 3.4D-E). Additionally, Ly49C^{+} NK cells from *B2m*$^{fl/fl}$ mice were preferentially responsive to stimulation through NK1.1, as indicated by a Ly49C licensing ratio above 1 (Fig. 3.4D, F). These baseline data show that NK cells from *B2m*$^{\Delta/\Delta}$ mice are unlicensed while NK cells from *B2m*$^{fl/fl}$ mice are licensed as previously shown for *B2m*$^{-/-}$ and WT NK cells (Kim et al., 2005).

Remarkably, however, NK cells from tamoxifen-treated *B2m*$^{fl/fl}$ R26-Cre-ER^{T2} mice exhibited reduced IFN-γ production in response to anti-NK1.1 stimulation (Fig. 3.4D-E). Additionally, NK cells from tamoxifen-treated *B2m*$^{fl/fl}$ R26-Cre-ER^{T2} mice exhibited a Ly49C licensing ratio below 1 (Fig. 3.4D, F). By contrast, control NK cells from *B2m*$^{fl/fl}$ R26-Cre-ER^{T2} mice pre-treated with vehicle control produced IFN-γ at levels similar to *B2m*$^{fl/fl}$ mice. Importantly, NK cells from tamoxifen-treated *B2m*$^{fl/fl}$ R26-Cre-ER^{T2} mice produced IFN-γ at levels comparable to controls in response to stimulation with PMA and ionomycin, which

bypasses activation receptor triggering (Fig. 3.4G). These data indicate that NK cells remain capable of producing IFN-γ after global MHC-I downregulation, but they become hypo-responsive to stimulation through activation receptors, i.e., they become unlicensed. Thus global downregulation of MHC-I induces NK cell tolerance to missing-self through resetting NK cell education.

3.2.4 Missing-self is observed after downregulation of MHC-I on transferred cells

In vivo evidence for the missing-self hypothesis has relied on transferred bone marrow (Bix et al., 1991), splenocytes (Oberg et al., 2004), and tumor cell lines (Kärre et al., 1986). To test if downregulation of MHC-I on transferred cells results in a distinct NK cell response compared to global downregulation of MHC-I, we adoptively transferred splenocytes from *B2m*$^{fl/fl}$ R26-Cre-ER^{T2} mice into WT recipients that were subsequently treated with tamoxifen or vehicle control (Fig. 3.6A). Remarkably, tamoxifen treatment resulted in only a small population of H-2K^{b}-deficient transferred cells (Fig. 3.6B-C). However, depletion of NK cells with anti-NK1.1 dramatically increased the percentage of H-2K^{b}-deficient transferred cells (Fig. 3.6B-C), indicating NK cell-mediated selection for residual MHC-I^{+} cells with tamoxifen treatment in mice not receiving anti-NK1.1 depletion. To further validate this interpretation, we directly compared co-transferred *B2m*$^{fl/fl}$ R26-Cre-ER^{T2} splenocytes and control *B2m*$^{fl/fl}$ splenocytes after tamoxifen treatment and found selective loss of *B2m*$^{fl/fl}$ R26-Cre-ER^{T2} splenocytes that was abrogated with NK cell depletion (Fig. 3.6D-E). These data indicate that downregulation of MHC-I on transferred cells is sufficient to induce missing-self recognition by NK cells. Because tamoxifen did not induce overt NK cell reactivity in *B2m*$^{fl/fl}$ R26-Cre-ER^{T2} mice (Fig. 3.3A-B), these results indicate that MHC-I downregulation is capable of inducing distinct NK cell

responses, cytotoxicity or adaptation, depending on the context, and that adoptively transferred cells are susceptible to missing-self rejection, even if they expressed MHC-I when transferred.

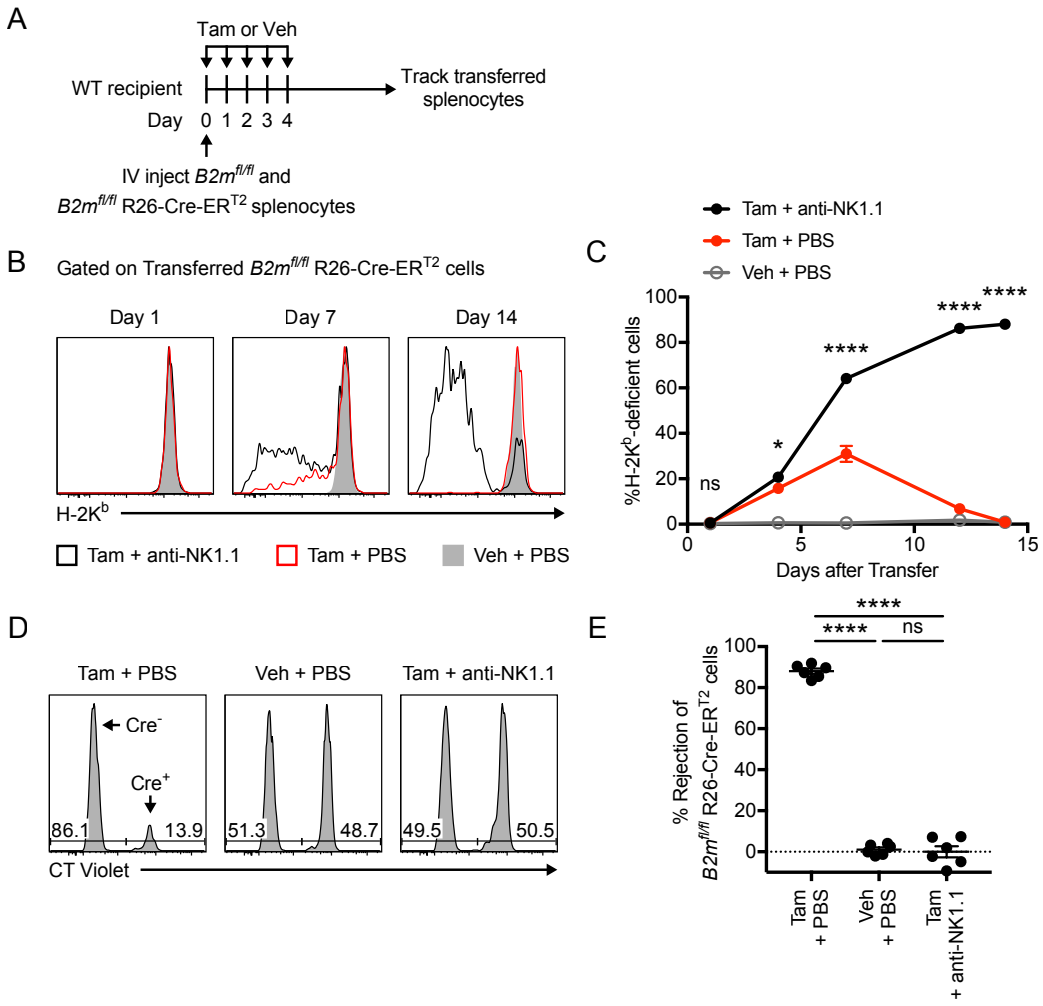


Figure 3.6: Downregulation of MHC-I on transferred cells induces missing-self susceptibility. (A) Splenocytes from *B2m*$^{fl/fl}$ and *B2m*$^{fl/fl}$ R26-Cre-ER^{T2} mice were labeled with CellTrace (CT) Far Red and differentially labeled with CT Violet and IV injected into WT recipient mice (20x10^6 of each donor). Recipient mice were then treated with tamoxifen (Tam) or vehicle control (Veh) for 5 days starting on the day of transfer. Recipient mice were injected IP with anti-NK1.1 to deplete NK cells or with PBS control as indicated. (B) Representative histograms showing H-2K^b expression on transferred *B2m*$^{fl/fl}$ R26-Cre-ER^{T2} cells (CT Far Red$^+$ CT Violet^{high}) from blood of recipient mice over time. (C) Percent of transferred *B2m*$^{fl/fl}$ R26-

Cre-ER^{T2} cells that are H-2K^{b}-deficient in the blood of recipient mice over time (n = 6 mice per group; two-way ANOVA). Asterisks in (C) indicate statistical significance between the tamoxifen-treated groups treated with anti-NK1.1 or PBS. (D) Representative histograms showing the relative percentages of transferred cells (CT Far Red^{+}) recovered from the spleens of recipient mice on day 14. *B2m*$^{fl/fl}$ (Cre^{-}) and *B2m*$^{fl/fl}$ R26-Cre-ERT2 (Cre^{+}) donor cells were distinguished by CT Violet labeling as indicated. (E) Percent NK cell-specific rejection of *B2m*$^{fl/fl}$ R26-Cre-ER^{T2} donor cells by WT recipient mice treated as indicated (n = 6 mice per group; one-way ANOVA). Data in (C) and (E) are combined from 2 independent experiments. Error bars indicate mean ± SEM.

We hypothesized that the number of cells that downregulate MHC-I might regulate whether NK cells adapt to or kill MHC-I-deficient cells. To test this, we bred *B2m*$^{fl/fl}$ mice to CD4-Cre-ER^{T2} transgenic mice to facilitate tamoxifen-induced deletion only in CD4$^+$ T cells (Aghajani et al., 2012). CD4$^+$ T cells were chosen as target cells because they constitute a substantially smaller cell population compared to those targeted by the R26-Cre-ER^{T2}, CD4$^+$ T cells have been suggested to be targets for NK cell cytotoxicity (Lu et al., 2007; Waggoner et al., 2011), and the CD4-Cre-ER^{T2} does not target NK cells (Fig. 3.7D-E). Treatment of *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2} mice with tamoxifen induced a subtle but reproducible reduction in the percent of CD4$^+$ T cells; however, we did not observe a statistically significant reduction in CD4$^+$ T cell number compared to tamoxifen-treated *B2m*$^{fl/fl}$ mice (Fig. 3.7A-C). These results suggest that downregulation of MHC-I on CD4$^+$ T cells leads to low-level missing-self recognition, supporting the idea that the number of cells that downregulate MHC-I may partially regulate the NK cell response. However, the majority of CD4$^+$ T cells that downregulated H-2K^b in response to tamoxifen were not rejected by NK cells (Fig. 3.7D-E), suggesting that NK cells establish tolerance to the remaining MHC-I-deficient CD4$^+$ T cells. To test this idea, we assessed whether *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2} mice pre-treated with tamoxifen were capable of rejecting transferred *B2m*$^{\Delta/\Delta}$ cells. *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2} mice treated with vehicle control were able to reject *B2m*$^{\Delta/\Delta}$ splenocytes to a similar degree as *B2m*$^{fl/fl}$ controls (Fig. 3.7F-G), indicating that *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2} NK cells were not tolerant to missing-self at baseline. However, tamoxifen-treatment abrogated the ability of NK cells in *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2} mice to reject *B2m*$^{\Delta/\Delta}$ splenocytes (Fig. 3.7F-G). These results show that downregulation of MHC-I only on CD4$^+$ T cells induces NK cell tolerance towards missing-self.

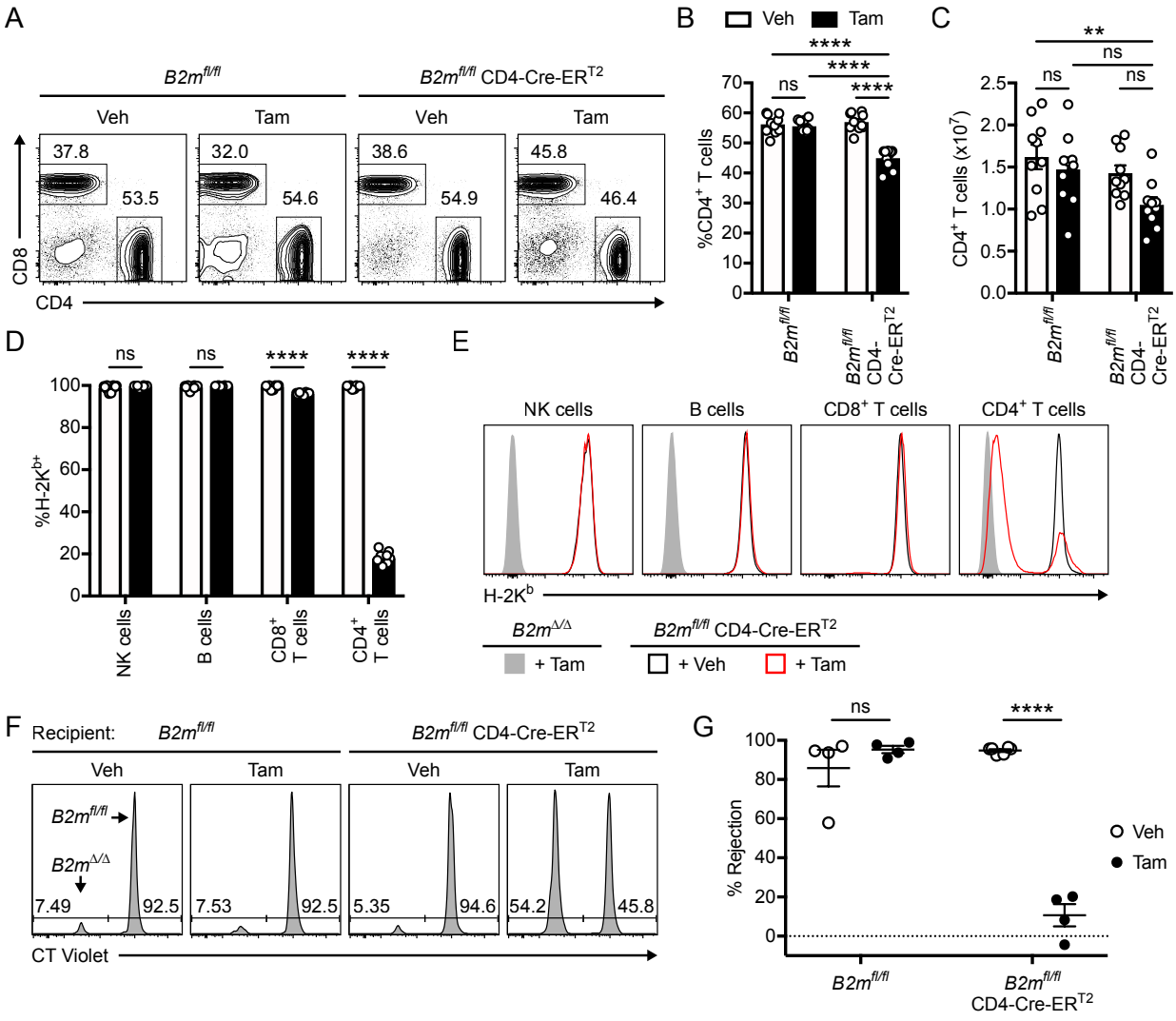


Figure 3.7: Minimal NK cell reactivity is observed after downregulation of MHC-I on CD4$^+$ T cells. *B2m$^{fl/fl}$* and *B2m$^{fl/fl}$* CD4-Cre-ER^{T2} mice were treated with tamoxifen (Tam) or vehicle control (Veh) on days 0 through 4, and spleens were harvested for flow cytometry on day 14. (A) Representative dot plots showing the percentage of splenic T cells (CD45$^+$ CD19$^-$ CD3$^+$) that express CD4 or CD8. (B) Percent of splenic T cells that express CD4 (n = 10 mice per group). (C) Number of CD4$^+$ splenic T cells (n = 10 mice per group). (D) Percent of NK cells (CD45$^+$ CD19$^-$ CD3$^-$ NK1.1$^+$ NKp46$^+$), B cells (CD45$^+$ CD3$^-$ CD19$^+$), CD8$^+$ T cells, and CD4$^+$ T cells

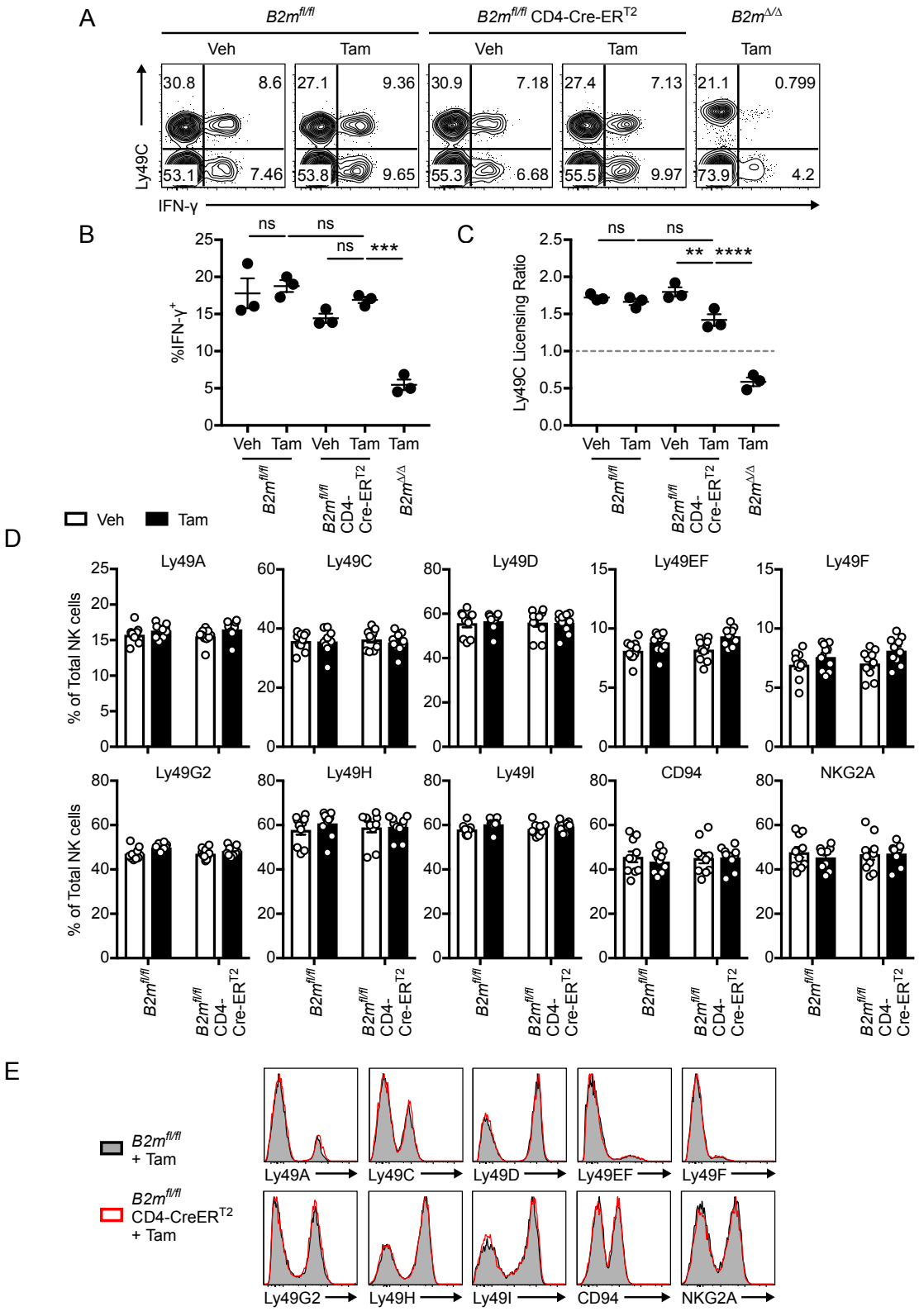
that express H-2K^{b} after treatment with tamoxifen (closed bars) or vehicle (open bars) (n = 10 mice per group). Data in (B-D) are combined from 3 independent experiments. (E) Representative histograms showing H-2K^{b} expression on the indicated cell types. (F-G) *B2m*$^{fl/fl}$ and *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2} mice were treated with tamoxifen or vehicle control on days 0 through 4 followed by IV injection of labeled donor splenocytes on day 14. Recipient spleens were harvested after 2 days to analyze donor cell recovery. (F) Representative histograms showing the relative percentages of transferred cells (CFSE^{+}) recovered from the spleens of recipient mice. (G) Summary of NK cell-specific rejection of *B2m*$^{\Delta/\Delta}$ donor cells (n = 4-6 mice per group). Data in (G) is combined from 2 independent experiments. Statistical significance was calculated by two-way ANOVA. Error bars indicate mean ± SEM.

3.2.5 NK cell tolerance to MHC-I-deficient CD4$^+$ T cells is not due to education

To further investigate the mechanism by which NK cells establish tolerance to MHC-I-deficient CD4$^+$ T cells in *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2} mice treated with tamoxifen, we stimulated splenocytes from *B2m*$^{fl/fl}$, *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2}, and *B2m*$^{\Delta/\Delta}$ mice with plate-bound anti-NK1.1. A higher percentage of NK cells from tamoxifen-treated *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2} mice produced IFN-γ after anti-NK1.1 stimulation as compared to *B2m*$^{\Delta/\Delta}$ mice (Fig. 3.8A-B). Importantly, NK cells from tamoxifen-treated *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2} mice produced IFN-γ at similar levels to *B2m*$^{fl/fl}$ mice and vehicle controls. This was accompanied by a Ly49C licensing ratio above 1 in tamoxifen-treated *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2} mice, indicating that NK cells remained licensed through Ly49C (Fig. 3.8C). We did notice a subtle reduction in the Ly49C licensing ratio comparing vehicle- and tamoxifen-treated *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2} mice in some experiments, but this is of unclear significance because the licensing ratio is still above 1 (Fig. 3.8C). Together these data show that NK cells remain responsive to stimulation through activation receptors after downregulation of MHC-I on CD4$^+$ T cells. These results suggest that MHC-I downregulation on CD4$^+$ T cells does not induce the same resetting of NK cell education that was seen after global downregulation of MHC-I. This suggests that distinct mechanisms establish NK cell tolerance to missing-self in *B2m*$^{fl/fl}$ R26-Cre-ER^{T2} and *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2} mice treated with tamoxifen.

We next tested if NK cells from tamoxifen-treated *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2} mice establish tolerance to MHC-I-deficient CD4$^+$ T cells through skewing of the inhibitory receptor repertoire. We observed similar expression of Ly49 receptors and CD94/NKG2A on NK cells from tamoxifen-treated *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2} mice compared to vehicle-treated or *B2m*$^{fl/fl}$ mice (Fig. 3.8D-E). Additionally, we did not observe any alterations in NK cell number or maturation based on expression of CD11b, CD27, and KLRG1 in tamoxifen-treated *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2} mice

(Fig. 3.9). These data suggest that NK cell tolerance to missing-self after MHC-I downregulation on $CD4^+$ T cells does not result from mechanisms analogous to T cell anergy or clonal deletion, which establish self-tolerance by rendering T cells hypo-responsive or by altering the T cell receptor repertoire respectively (Xing and Hogquist, 2012).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B2m fl/fl
B2m fl/fl CD4-Cre-ERT2
B2m Δ/Δ
Veh
Tam
Ly49C
IFN-γ
30.8
8.6
53.1
7.46
27.1
9.36
53.8
9.65
30.9
7.18
55.3
6.68
27.4
7.13
55.5
9.97
21.1
0.799
73.9
4.2
B
%IFN-γ+
ns
***
C
Ly49C Licensing Ratio
**
****
D
% of Total NK cells
Ly49A
Ly49C
Ly49D
Ly49EF
Ly49F
Ly49G2
Ly49H
Ly49I
CD94
NKG2A
E
B2m fl/fl + Tam
B2m fl/fl CD4-CreER T2 + Tam

Figure 3.8: NK cell responsiveness and receptor repertoire are not affected by MHC-I downregulation on CD4$^+$ T cells. *B2m$^{fl/fl}$*, *B2m$^{fl/fl}$* CD4-Cre-ER^{T2}, and *B2m$^{\Delta/\Delta}$* mice were treated with tamoxifen (Tam) or vehicle control (Veh) on days 0 through 4, and splenocytes were harvested on day 14 for anti-NK1.1 stimulation and receptor repertoire analysis. (A) Representative dot plots showing the percentage of total NK cells (CD45$^+$ CD3$^-$ CD19$^-$ NKp46$^+$) that express Ly49C and IFN-γ after stimulation with plate-bound anti-NK1.1. (B) Summary of the percentage of total NK cells that express IFN-γ after stimulation with plate-bound anti-NK1.1 (n = 3 mice per group; one-way ANOVA). (C) The Ly49C licensing ratio for total NK cells after stimulation with anti-NK1.1 (n = 3 mice per group; one-way ANOVA). Data in (B) and (C) are representative of 3 independent experiments with 3-4 mice per group. (D) The percentage of NK cells (CD3$^-$ CD19$^-$ NK1.1$^+$ NKp46$^+$) that express the indicated receptor (n = 10 mice per group). Data in (D) are combined from 3 independent experiments. (E) Representative histograms showing expression of the indicated receptor on NK cells from *B2m$^{fl/fl}$* and *B2m$^{fl/fl}$* CD4-Cre-ER^{T2} mice treated with tamoxifen. Error bars indicate mean ± SEM.

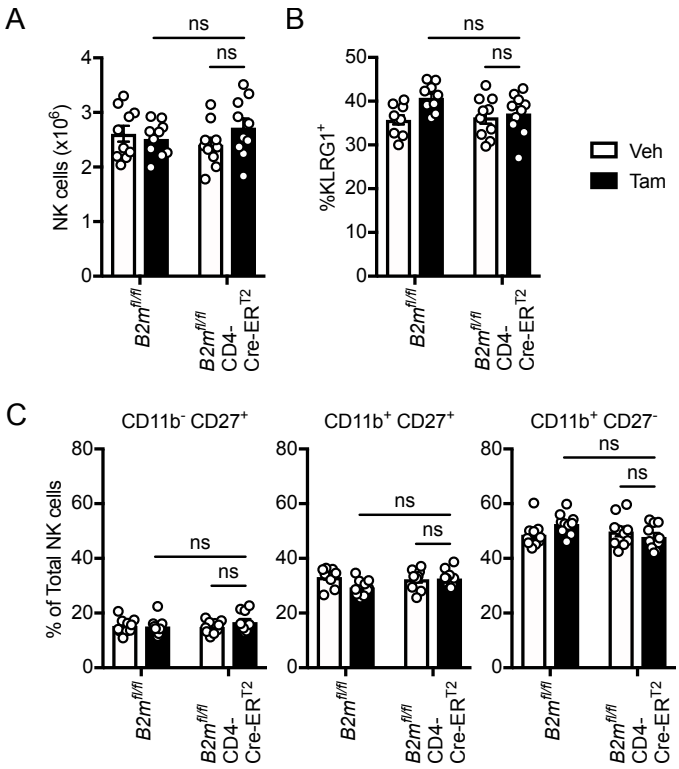

Figure 3.9: No changes in NK cell development or maturation are observed after downregulation of MHC-I on CD4$^+$ T cells. *B2m$^{fl/fl}$* and *B2m$^{fl/fl}$* CD4-Cre-ER^{T2} mice were treated with tamoxifen (Tam) or vehicle control (Veh) on days 0 through 4, and splenocytes were harvested on day 14 for flow cytometry. (A) Total number of splenic NK cells (CD3$^-$ CD19$^-$ NK1.1$^+$ NKp46$^+$) from the indicated mice treated with vehicle (open bars) or tamoxifen (closed bars) (n = 10 mice per group). (B) The percentage of total NK cells that express KLRG1 (n = 10 mice per group). (C) The percentage of total NK cells in different maturation stages separated by expression of CD11b and CD27 (n = 10 mice per group). Data are combined from 3 independent experiments. Statistical significance was calculated by two-way ANOVA. Error bars indicate mean ± SEM.

3.2.6 Robust missing-self reactivity occurs in the context of infection

Previous studies have shown that MCMV infection breaks NK cell tolerance to missing-self in mixed WT:*B2m*$^{-/-}$ chimeras (Shifrin et al., 2016; Sun and Lanier, 2008a). This led us to hypothesize that viral infection induces NK cell missing-self reactivity in response to acute downregulation of MHC-I as well. To test this idea, *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2} mice were treated with tamoxifen or vehicle control and subsequently infected with Δm157 MCMV (Fig. 3.10A). Mice were infected with Δm157 instead of WT MCMV to exclude the effects of Ly49H-mediated cytotoxicity and so that NK cells could be depleted without significantly increasing the viral burden (Brown et al., 2001; Bubic et al., 2004; Parikh et al., 2015), which could have confounded previous experiments with mixed chimeras (Sun and Lanier, 2008a). In the context of NK cell depletion, CD4$^+$ T cells from tamoxifen-treated *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2} mice were mostly MHC-I-deficient even in the context of infection (Fig. 3.10B). Remarkably, however, a high percentage of CD4$^+$ T cells in undepleted *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2} mice treated with tamoxifen and infected with Δm157 MCMV expressed MHC-I (Fig. 3.10B). This selection for MHC-I-positive cells was accompanied by a reduction in the number of splenic CD4$^+$ T cells in tamoxifen-treated *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2} mice infected with Δm157 MCMV compared to NK cell-depleted mice and *B2m*$^{fl/fl}$ controls (Fig. 3.10C). These results together suggest that in the context of Δm157 MCMV infection, NK cells robustly reject CD4$^+$ T cells that downregulate MHC-I.

3.2.7 Pattern recognition receptor agonist stimulation drives NK cell reactivity towards missing-self

Previous studies have shown that cytotoxic control of MCMV requires type I interferon and IL-12 to prime NK cells by promoting expression of perforin and granzyme B (Fehniger et al., 2007; Parikh et al., 2015) but it is unclear if such priming plays a role in NK cell self-

tolerance. We hypothesized that NK cell missing-self reactivity in tamoxifen-treated *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2} mice during viral infection might be regulated by NK cell priming by inflammatory cytokines instead of NK cell education. To test the role of inflammation in missing-self recognition *in vivo*, *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2} mice were treated with tamoxifen followed by PBS or poly(I:C), an adjuvant that signals through the pattern recognition receptors (PRRs) TLR3 and MDA5 to induce production of inflammatory cytokines that activate NK cells (Akazawa et al., 2007; McCartney et al., 2009). Poly(I:C) thus mimics aspects of the inflammatory environment induced by MCMV infection, which also triggers MyD88-dependent responses (Krug et al., 2004). Remarkably, poly(I:C) induced rapid loss of CD4$^+$ T cells from the peripheral blood of *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2} but not *B2m*$^{fl/fl}$ mice treated with tamoxifen (Fig. 3.10D-E). This rapid loss of CD4$^+$ T cells was not observed in *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2} mice treated with tamoxifen and PBS control (Fig. 3.10D-E). Importantly, depletion of NK cells with anti-NK1.1 completely abrogated the loss of CD4$^+$ T cells induced by poly(I:C) (Fig. 3.10D-E). These data indicate that poly(I:C) induces NK cell missing-self reactivity in tamoxifen-treated *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2} mice.

To test if poly(I:C) acts through potentiating missing-self recognition, expression of MHC-I was analyzed on residual CD4$^+$ T cells from the spleens of *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2} mice treated with tamoxifen and poly(I:C) (Fig. 3.10F-G). Tamoxifen induced downregulation of H-2K^b on ~80% of splenic CD4$^+$ T cells from *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2} mice treated with tamoxifen and PBS control. Remarkably, substantially higher percentages of CD4$^+$ T cells from *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2} mice treated with tamoxifen and poly(I:C) expressed H-2K^b compared to mice that did not receive poly(I:C) (Fig. 3.10F-G). In contrast, depletion of NK cells with anti-NK1.1 completely abrogated this effect (Fig. 3.10F-G), indicating that poly(I:C) induced an NK cell-

dependent selection for MHC-I^{+} cells that was accompanied by an NK cell-dependent reduction in splenic CD4^{+} T cell number in *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2} mice treated with tamoxifen and poly(I:C) (Fig. 3.10H). These data show that poly(I:C) induces NK cell reactivity towards MHC-I-deficient CD4^{+} T cells, indicating that a PRR agonist can regulate NK cell reactivity towards missing-self.

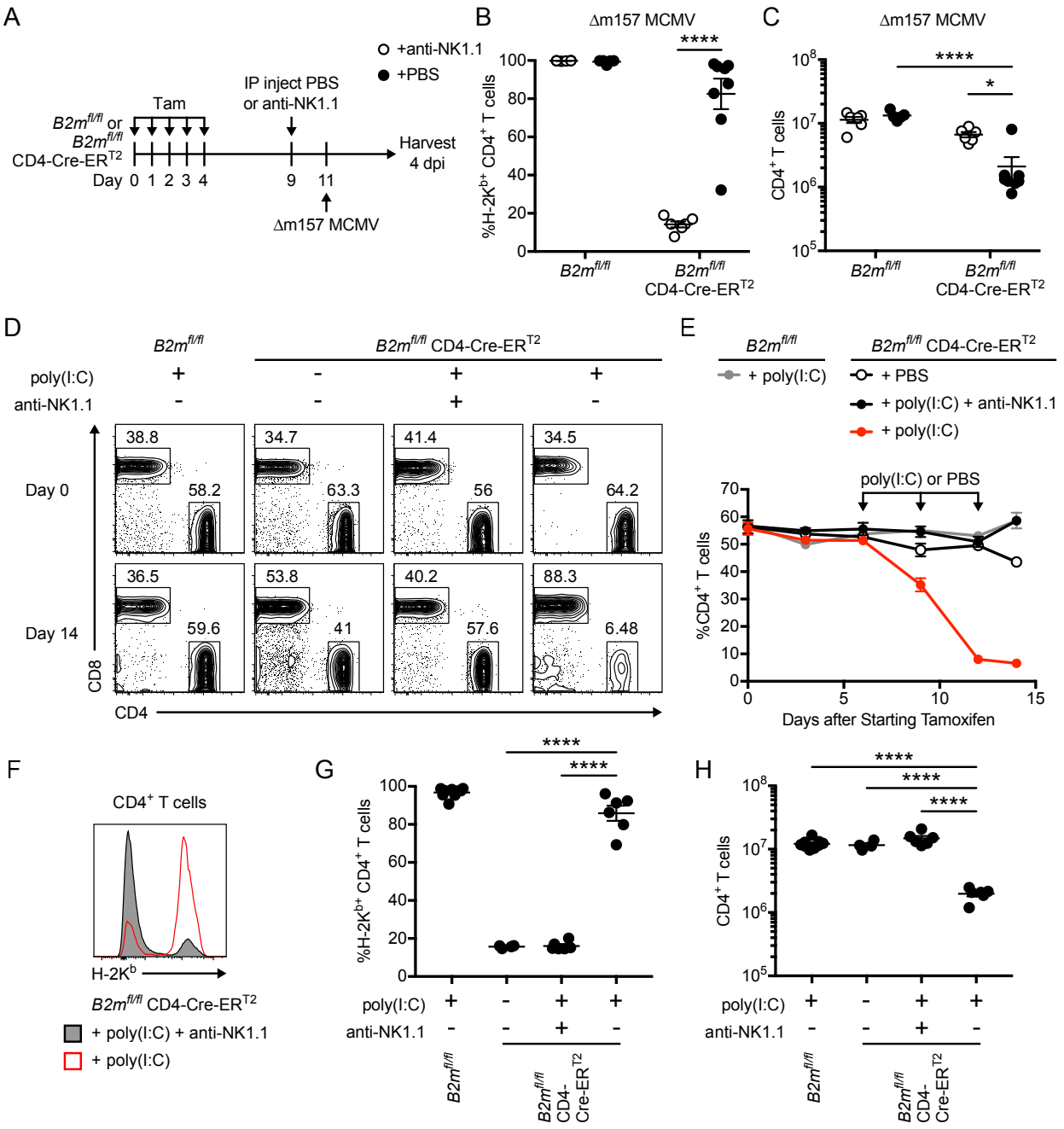

Figure 3.10: MCMV infection and poly(I:C) induce missing-self reactivity towards MHC-I-deficient CD4$^+$ T cells. (A-C) *B2m$^{fl/fl}$* and *B2m$^{fl/fl}$* CD4-Cre-ER^{T2} mice were treated with tamoxifen (Tam) on days 0-4, injected with PBS or anti-NK1.1 antibody on day 9, and infected with 5x10^3 PFU of Δm157 MCMV on day 11. Splenocytes were harvested 4 days post infection

for flow cytometry as depicted in the schematic in (A). (B) The percentage of $CD4^+$ T cells ($CD45^+$ $CD19^-$ $CD3^+$ $CD8^-$ $CD4^+$) that express H-$2K^b$ in the spleens of mice 4 days post infection (n = 5-8 mice per group; two-way ANOVA). (C) The number of splenic $CD4^+$ T cells 4 days post infection (n = 5-8 mice per group; two-way ANOVA). (D-H) *B2m*$^{fl/fl}$ and *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2} mice were treated with tamoxifen or vehicle control on days 0 through 4 followed by IP injection of poly(I:C) or PBS on days 6, 9, and 12. Mice were injected with anti-NK1.1 to deplete NK cells or with PBS control on day -2 and every 7 days after. (D) Representative dot plots showing CD4 and CD8 expression on peripheral blood T cells ($CD45^+$ $CD19^-$ $CD3^+$) on days 0 and 14. (E) Percentage of blood T cells that express CD4 over time (n = 4-7 mice per group). (F) Representative histogram showing H-$2K^b$ expression on day 14 splenic $CD4^+$ T cells from anti-NK1.1- or PBS-treated *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2} mice treated with tamoxifen and poly(I:C). (G) Percentage of splenic $CD4^+$ T cells that express H-$2K^b$ on day 14 (n = 4-9 mice per group; one-way ANOVA). (H) Number of splenic $CD4^+$ T cells on day 14 (n = 4-9 mice per group; one-way ANOVA). Data in (B), (C), (E), (G), and (H) are combined from 2 independent experiments. Error bars indicate mean ± SEM.

3.3 Discussion

Here we show that acute downregulation of MHC-I *in vivo* does not automatically lead to NK cell-mediated rejection, as predicted by the missing-self hypothesis. Instead, acute loss of MHC-I can result in multiple distinct NK cell responses. Global downregulation of MHC-I was found to induce NK cell tolerance to missing-self by resetting NK cell education. In contrast, downregulation of MHC-I on transferred cells or $CD4^+$ T cells could trigger missing-self recognition and cytotoxicity but this was limited to only a few cells. Additionally, $CD4^+$ T cell-specific downregulation of MHC-I was able to induce NK cell tolerance to missing-self without evidence of an alteration in the capacity of NK cells to be triggered through their activation receptors. In this setting, viral infection and PRR agonist stimulation could drive robust NK cell reactivity towards missing-self. Thus, our findings suggest that inflammation is important for breaking NK cell self-tolerance, despite the generally accepted paradigm based on the missing-self hypothesis alone.

3.3 Materials and Methods

3.3.1 Mice

C57BL/6 (B6) mice were purchased from Charles River Laboratories (Wilmington, MA). *B2m*$^{-/-}$ (B6.129P2-*B2m*^{tm1Unc}/J), Albino B6 (B6(Cg)-*Tyr*$^{c-2J}$/J), Rosa26-Cre-ER^{T2} (B6.129-*Gt(ROSA)26Sor*$^{tm1(cre/ERT2)Tyj}$/J), and CD4-Cre-ER^{T2} (B6(129X1)-Tg(Cd4-cre/ERT2)11Gnri/J) mice were purchased from The Jackson Laboratory. FLPe transgenic mice (C57BL/6-Tg(CAGGS-Flpe)2ARTE) were purchased from Artemis Pharmaceuticals. CMV-Cre mice (Schwenk et al., 1995) on the B6 background were provided by Marco Colonna (Washington University, St. Louis, MO). Mice were 8-13 weeks old at the start of experiments except mice

used for donor splenocytes in *in vivo* cytotoxicity assays, which were 8-18 weeks old at the time of transfer.

3.3.2 Development of floxed *B2m* mice

Three JM8A3.N1 (C57BL/6N-A^{tm1Brd}) ES cell clones (HEPD0673_4_D09, HEPD0673_4_G10, and HEPD0673_4_H09) carrying the targeted allele *B2m*$^{tm1a(EUCOMM)Hmgu}$ (*B2m*^{tm1a}) were purchased from the European Conditional Mouse Mutagenesis Consortium (EUCOMM). Clone HEPD0673_4_D09 was confirmed to be correctly targeted by Southern blot analysis and was microinjected into Albino B6 blastocysts by the Transgenic, Knockout, and Micro-Injection Core at Washington University in St. Louis. Chimeric mice were bred to Albino B6 to identify germline transmission by coat color. Mice containing the germline-transmitted *B2m*^{tm1a} allele were subsequently bred to FLPe transgenic mice to generate the *B2m*^{tm1c} (*B2m*^{fl}) allele. The albino and FLPe alleles were removed through breeding. Mice carrying the *B2m*^{fl} allele were bred to CMV-Cre transgenic mice to generate the *B2m*^{tm1d} (*B2m*$^{\Delta}$) allele with a germline-deletion of *B2m*. The CMV-Cre transgene was subsequently bred out prior to experiments with *B2m*$^{\Delta/\Delta}$ mice except in one replicate of the experiment in Fig. 3.1E-F, in which the *B2m*$^{\Delta/\Delta}$ donor cells were heterozygous for CMV-Cre.

3.3.3 Antibodies and flow cytometry

The following were purchased from BD Biosciences: anti-Ly49G2 (4D11), anti-Ly49F (HBF-719), streptavidin PE. The following were purchased from eBioscience: Fixable Viability Dye eFluor 506, Fixable Viability Dye eFluor 780, anti-IFNγ (XMG1.2), anti-NKp46 (29A1.4), anti-NK1.1 (PK136), anti-CD19 (eBio1D3), anti-CD3e (145-2C11), anti-CD11b (M1/70), anti-CD45 (30-F11), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD4 (RM4-5), anti-CD8b (eBioH35-17.2), anti-CD8a (53-6.7), anti-Ly49H (3D10), anti-Ly49E/F (CM4), anti-Ly49D (eBio4E5),

anti-Ly49G2 (eBio4D11), anti-NKG2AB6 (16a11), anti-CD94 (18d3), anti-CD27 (LG.7F9). The following were purchased from BioLegend: streptavidin APC, anti-KLRG1 (2F1), anti-NK1.1 (PK136), anti-H-2K^b (AF6-88.5), anti-H-2D^b (KH95). The following was purchased from Abcam: anti-Ly49I (YLI-90). The following was purchased from ThermoFisher: anti-Ly49F (HBF-719). The following was purchased from Jackson ImmunoResearch: Alexa Fluor 647-conjugated Goat anti-Mouse IgGγ, Fc subclass 3 specific (anti-mouse IgG3). PK136 antibody (anti-NK1.1) was purified in our laboratory from supernatants of the PK136 hybridoma (American Type Culture Collection, Manassas, VA). NK cell depletion was performed by intraperitoneal (IP) injection of 200μg of purified PK136 antibody. Mice were excluded from analysis if PK136 injection was shown to be unsuccessful by flow cytometry for NK cells in the blood after the first injection or in the spleen. JR9 antibody (anti-Ly49A) was purified in our laboratory from supernatants of the JR9 hybridoma that was provided by Jacques Roland (Pasteur Institute, Paris, France). 4LO3311 antibody (anti-Ly49C) was purified in our laboratory from supernatants of the 4LO3311 hybridoma that was provided by Suzanne Lemieux (Institut National de la Recherche Scientifique-Institut Armand-Frappier, Laval, Quebec, Canada). Anti-Ly49A and anti-Ly49C antibodies were conjugated in our laboratory to FITC or to biotin using the EZ-Link Sulfo-NHS-LC-LC-biotin kit (Thermo Fisher). Ly49C staining was performed with either anti-Ly49C-biotin followed by fluorophore-conjugated streptavidin or purified anti-Ly49C followed by anti-mouse IgG3-Alexa Fluor 647. Blocking of Fc receptors was performed with supernatants of the 2.4G2 (anti-FcγRII/III) hybridoma (American Type Culture Collection, Manassas, VA). Surface staining for flow cytometry was performed on ice in either 2.4G2 supernatant or staining buffer (PBS with 1% BSA and 0.01% Sodium Azide). Cells were gated on lymphocytes with exclusion of doublets and exclusion of dead cells with Fixable Viability

Dye (BD Biosciences) or FSC and SSC (only in some *in vivo* cytotoxicity experiments). $CD45^+$ cells throughout the text refers to cells stained with either the anti-CD45 or anti-CD45.2 antibody. $CD8^+$ cells throughout the text refers to cells stained with either the anti-CD8a or anti-CD8b antibodies. Samples were analyzed using a FACSCanto (BD Biosciences) or a FACSAria Fusion (BD Biosciences). Data was analyzed using FlowJo v9.9.6 or v10.4.2 (Tree Star, Ashland, OR).

3.3.4 Tamoxifen treatment of mice

Mice were administered tamoxifen (Sigma-Aldrich) by oral gavage (4mg/day for 5 consecutive days) in 200µl of corn oil (Sigma-Aldrich) as according to published protocols (Anastassiadis et al., 2010). In most experiments, NK cells were depleted from a subset of mice by intraperitoneal (IP) injection of 200µg anti-NK1.1 (PK136) antibody on day -2 and every 7 days after for the duration of the experiment. When indicated, 100µg of poly(I:C) HMW (InvivoGen) in 0.9% NaCl or a PBS control was administered to mice by IP injection.

3.3.5 *In vivo* cytotoxicity assay

Donor splenocytes were harvested and labeled *in vitro* with different combinations of 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE; Life Technologies), CellTrace Violet (CT Violet; Thermo Fisher Scientific), and CellTrace Far Red (CT Far Red; Thermo Fisher Scientific). For experiments with 6 donor cell populations, all donor cells were labeled with 2.5µM CFSE, and donors were differentially labeled with CT Violet (5µM, 1µM, or 0.2µM) and CT Far Red (1µM or 0.04µM). For experiments with 4 donor cell populations, all donor cells were labeled with 2.5µM CFSE, and donors were differentially labeled with CT Violet (5µM or 0.2µM) and CT Far Red (1µM or 0.04µM). For experiments with 2 donor cell populations, all donor cells were labeled with either 2.5µM CFSE or with 0.2µM CT Far Red depending on the

experiment. The 2 donor cell populations were differentially labeled with CT Violet (5μM or 0.2μM). In some experiments, 4 donor cell populations were injected, but only 2 were displayed in the figure for clarity. Recipient mice were injected IV with $2x10^6$ of each donor cell except for Fig. 3.6, in which $20x10^6$ of each donor cell were injected. Spleens from recipient mice were harvested 2 days after transfer of donor cells except for Fig. 3.6. NK cell-specific rejection was calculated by gating on transferred cells and excluding dead cells either by Fixable Viability Dye (BD Biosciences) or FSC and SSC. Rejection was quantified as %Rejection = [1-(Target/Control)/$(Target/Control)_{Average(NK\text{-}depleted)}$] x 100 where the target was the donor cell of interest and the control was a WT or $B2m^{fl/fl}$ donor cell population depending on the experiment. The ratio of target-to-control cells is normalized to the average ratio recovered from NK cell-depleted mice to calculate rejection by NK cells.

3.3.6 *In vitro* splenocyte stimulations

Splenocytes were stimulated *in vitro* as previously described (Jonsson and Yokoyama, 2010; Kim et al., 2005). Briefly, $5x10^6$ splenocytes in R10 media were plated into 24-well culture plates that that were pre-coated with anti-NK1.1 (PK136; 1μg/mL) or that contained 0.5μg/mL PMA (Sigma-Aldrich) and 4μg/mL ionomycin (Sigma-Aldrich). Cells were incubated for a total of 7 hours at 37°C. GolgiPlug (BD Biosciences) was added to the wells after the first hour. After stimulation, cells were stained with fixable viability dye and subsequently with antibodies to surface antigens. Cells were then fixed and permeabilized using the Fixation/Permeabilization Solution Kit (BD Cytofix/Cytoperm) to stain for intracellular IFN-γ. Fixable viability dye was used to gate on viable cells in all experiments. The Ly49C licensing ratio was calculated as follows (Jonsson and Yokoyama, 2010): Ly49C licensing ratio = [(%$Ly49C^+IFN\text{-}\gamma^+$)/(%$Ly49C^+$)]/[(%$Ly49C^-IFN\text{-}\gamma^+$)/(%$Ly49C^-$)].

3.3.7 MCMV infections

Infections were performed with $5x10^3$ PFU of Δm157 MCMV, which was previously shown to differ from the WT1 MCMV strain by a single nucleotide (Cheng et al., 2010; Parikh et al., 2015). Mice were infected by IP injection of salivary gland propagated virus in 200µl PBS.

3.3.8 Statistics

Statistical analysis was performed using Prism 7 (GraphPad, La Jolla, CA). *P*-values were calculated using either one-way ANOVA or two-way ANOVA with Bonferroni's multiple comparison's test. Asterisks indicate statistical significance as follows: **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns = not significant.

Chapter 4: Discussion

4.1 Ly49-MHC-I inhibition is not required for NK cell development or maintenance

B2m$^{-/-}$ mice and Ly49A-tg mice contain normal numbers of NK cells (Held et al., 1996a; Held and Raulet, 1997; Liao et al., 1991). In contrast, Ly49A-tg mixed chimeras suggest that Ly49A-H-2D^{d} signaling promotes NK cell development (Lowin-Kropf and Held, 2000), and an independent Ly49A-tg strain exhibits a complete block in NK cell development (Kim et al., 2000). Here we used AYF D8 KODO mice to directly show that endogenous Ly49A ITIM signaling is not required for development of normal numbers of NK cells or for their maturation. These results argue that NK cell development does not involve a selection step that is analogous to positive selection of TCRs that bind to self-MHC-I during T cell development (Xing and Hogquist, 2012). Additionally, NK cell development and maturation were not substantially affected by acute downregulation of MHC-I globally in tamoxifen-treated *B2m*$^{fl/fl}$ R26-Cre-ER^{T2} mice. These results suggest that NK cells do not require tonic interactions between Ly49s and MHC-I for survival in contrast to CD8^{+} T cells which require TCR-MHC-I interactions for survival (Tanchot et al., 1997). Because NK cell education is impaired in AYF D8 KODO mice and in tamoxifen-treated *B2m*$^{fl/fl}$ R26-Cre-ER^{T2} mice, these results also separate NK cell development from education.

4.2 Downregulation of the Ly49 MFI is not ITIM-dependent

Signaling induces endocytosis of many receptors such as G-protein-coupled receptors, which are internalized through a β-arrestin-dependent pathway (Perry and Lefkowitz, 2002). Although the presence of self-MHC-I ligand correlates with a reduction in the MFI of Ly49A

and Ly49C, it has been unclear whether this is due receptor internalization or epitope masking by binding to MHC-I in *cis* (Andersson et al., 2007; Held et al., 1996b). Additionally, β-arrestin 2 has been shown to signal downstream of KIR2DL1 to potentially alter receptor expression (Yu et al., 2008). The AYF D8 KODO mouse shows that Ly49A ITIM signaling is not required for H-2D^{d} to downregulate the Ly49A MFI. Surprisingly, however, downregulation of MHC-I expression on NK cells in tamoxifen-treated *B2m$^{fl/fl}$* R26-Cre-ER^{T2} mice did not upregulate the Ly49C MFI to the level seen in *B2m$^{\Delta/\Delta}$* mice (Fig. 3.4D). As a result, these findings suggest that the Ly49 MFI shift is not caused by ITIM signaling, but interactions between MHC-I and Ly49 may lead to long-lasting effects on Ly49 surface expression that are ITIM-independent.

4.3 Ly49 signaling controls receptor repertoire development

Although we did not observe an effect of the Ly49-MHC-I interaction on NK cell development, Ly49A ITIM signaling was found to control receptor repertoire development. Ly49 ITIM signaling was previously suggested to impact receptor repertoire development by comparing Ly49 expression on Ly49A^{BALB} and Ly49A-Y8F^{BALB} transgenic mice that express H-2D^{d} (Bessoles et al., 2013). These studies, however, utilized the BALB allele of Ly49A for which the ligand-specificity has not been as well characterized as the B6 allele studied here. Additionally, these studies relied on comparisons between two different Ly49A^{BALB} transgenic lines in which the Ly49A^{BALB} transgene was probably expressed at different levels and incorporated at different locations in the genome. Likely as a result of these caveats, the Ly49A^{BALB} transgene induced an ITIM-dependent increase in the percentage of Ly49F^{+} NK cells, which is opposite of the effects of Ly49A^{B6} transgene expression in H-2^{d} mice (Hanke et al., 2001) and of our findings in AYF D8 KODO mice. Here, we use the AYF D8 KODO mouse

to show that endogenous Ly49A^{B6} ITIM signaling influences the inhibitory receptor repertoire in a mouse with relatively normal expression of Ly49A.

Our results also show that the NK cell receptor repertoire subtly adjusts to acute global downregulation of MHC-I. Interestingly, these subtle changes were not observed after downregulation of MHC-I on CD4$^+$ T cells. Similarly, floxed H-2D^d transgenic mice crossed to CD4-Cre that constitutively lack H-2D^d only on T cells have been shown to exhibit a similar NK cell receptor repertoire as mice that globally express the H-2D^d transgene (Bessoles et al., 2013). These results along with ours suggest that MHC-I expression on specific cell types may be important for dynamically regulating the inhibitory receptor repertoire. It remains unclear, however, if only a subset of repertoire changes are reversible, whether this process involves Ly49 ITIM signaling, and which cell types express MHC-I to induce repertoire skewing.

The sequential model of repertoire development proposes that NK cells stochastically acquire Ly49 expression sequentially during development until a signaling threshold is reached (Raulet et al., 1997). In contrast, the two-step selection model proposes that a repertoire of NK cells develop that express stochastic combinations of Ly49s, and NK cells are selected that express some but not too many self-MHC-I-specific Ly49 receptors in analogy to T cell development (Raulet et al., 1997; Xing and Hogquist, 2012). The sequential model predicts that a higher percentage of Ly49A$^+$ NK cells from AYF D8 KODO mice should express each Ly49 receptor compared to Ly49A$^+$ NK cells from D8 KODO mice regardless of MHC-I specificity. According to the two-step selection model, a higher percentage of Ly49A$^+$ NK cells from AYF D8 KODO mice should express only the Ly49s that bind to H-2D^d. However, we observed that a higher percentage of Ly49A$^+$ NK cells from AYF D8 KODO mice expressed the H-2^d-specific receptors Ly49F, Ly49G2, and Ly49I but we did not observe a change in expression of the H-

2D^{d}-specific receptors Ly49C or Ly49D. As a result, the changes in the NK cell receptor repertoire in AYF D8 KODO compared to D8 KODO mice are not fully explained by either the sequential or the two-step selection model. Additionally, neither model explains the subtle changes in the receptor repertoire that we observed after global MHC-I downregulation in tamoxifen-treated *B2m*$^{fl/fl}$ R26-Cre-ER^{T2} mice. It is possible that our data suggest that the receptor repertoire is established though some combination of the sequential and two-step selection models or through a combination of developmental and peripheral mechanisms. Alternatively, our results may suggest that some of the MHC-I-binding specificities attributed to Ly49s by tetramer binding and cell-cell adhesion assays may not reflect physiologically relevant ligands.

4.4 NK cells remain self-tolerant in the absence of global Ly49-MHC-I inhibition

Although the missing-self hypothesis predicts that NK cells kill MHC-I-deficient self cells, MHC-I-deficient mice do not exhibit NK cell autoreactivity (Liao et al., 1991). It is possible, however, that NK cells remain self-tolerant in MHC-I-deficient mice because self-MHC-I-specific inhibitory Ly49s also bind to additional non-MHC-I ligands to prevent autoreactivity. Here we mutated the Ly49A ITIM to test if a loss of all inhibitory signaling through Ly49A would induce NK cell autoreactivity. Consistent with studies of MHC-I-deficient mice (Liao et al., 1991), we did not observe any obvious signs of autoimmunity in AYF D8 KODO mice. These results show that NK cells can establish self-tolerance even in the absence of inhibitory Ly49 signaling.

Although NK cells from MHC-I-deficient and AYF D8 KODO mice are self-tolerant, NK cells from WT mice are known to reject adoptively transferred MHC-I-deficient cells

without prior activation (Bix et al., 1991; Oberg et al., 2004). Based on these findings, we hypothesized that acute downregulation of MHC-I in a mouse with educated NK cells would induce missing-self reactivity. Surprisingly, however, we did not observe any overt signs of NK cell autoreactivity after global MHC-I downregulation in *B2m*$^{fl/fl}$ R26-Cre-ER^{T2} mice. We did, however, observe rejection of transferred *B2m*$^{fl/fl}$ R26-Cre-ER^{T2} splenocytes in tamoxifen-treated WT recipients. Additionally, we observed a subtle rejection of CD4^{+} T cells in tamoxifen-treated *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2} mice that was restricted to a small number of cells. These results show that NK cells exhibit a low-level ability to reject missing-self targets after acute downregulation of MHC-I on a small number of transferred cells or CD4^{+} T cells. However, downregulation of MHC-I largely induces NK cell tolerance to missing-self, similar to what is seen in *B2m*$^{-/-}$ and AYF D8 KODO mice.

Bone marrow transfer experiments have shown that high doses of missing-self targets can overcome NK cell-mediated rejection (Cudkowicz and Bennett, 1971; Koh et al., 2005). Similarly, it is possible that no NK cell autoreactivity was observed after global MHC-I downregulation because the large number of missing-self targets overwhelmed NK cell cytotoxicty. Alternatively, the NK cell response to missing-self may differ qualitatively depending on the dose of MHC-I-deficient cells. We favor the latter hypothesis due to the finding that NK cell responsiveness to stimulation differs after global MHC-I downregluation compared to MHC-I downregulation only on CD4^{+} T cells.

4.5 NK cell education is dynamically controlled by Ly49 signaling

Previous studies suggested that adoptively transferred WT NK cells adapt to an MHC-I-deficient environment by becoming re-educated and hypo-responsive to stimulation (Joncker et

al., 2010). However, *B2m*$^{-/-}$ recipient mice in these studies were irradiated or depleted of T cells to prevent rejection of MHC-I^{+} donor NK cells (Apasov and Sitkovsky, 1993; Glas et al., 1994; Joncker et al., 2010; Zijlstra et al., 1992). Here we use *B2m*$^{fl/fl}$ R26-Cre-ER^{T2} and *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2} mice to directly test how NK cells respond to changes in the MHC-I environment without adoptive transfers, irradiation, or T-cell-depletion, which could have confounded prior studies. Our results show that global MHC-I downregulation induces NK cells to adapt through becoming hypo-responsive to stimulation, similar to prior adoptive transfer experiments (Joncker et al., 2010). In contrast, NK cells remained responsive to stimulation after downregulation of MHC-I on only CD4^{+} T cells. These results show that global MHC-I downregulation leads to resetting of NK cell responsiveness, but NK cells can remain educated in environments in which MHC-I is downregulated on a limited number of cells, which more closely corresponds to the environment that NK cells encounter during early stages of infection and tumorigenesis.

Previous studies have shown that mixed WT:*B2m*$^{-/-}$ fetal liver chimeras are tolerant of *B2m*$^{-/-}$ bone marrow grafts (Wu and Raulet, 1997). These results have been used to support the disarming model of NK cell education because a small number of MHC-I-deficient cells dominantly induce tolerance to missing-self (Wu and Raulet, 1997; Yokoyama and Kim, 2006). Here we show that tamoxifen-treated *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2} mice are unable to reject MHC-I-deficient splenocytes, which also suggests that MHC-I-deficient cells dominantly induce tolerance to missing-self. Importantly, however, NK cells from tamoxifen-treated *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2} mice remain educated. Similarly, recent studies have shown that NK cells from WT:*B2m*$^{-/-}$ fetal liver chimeras remain responsive to stimulation despite being tolerant to missing-self (Shifrin et al., 2016). As a result, our findings do not support the disarming model of

NK cell education but instead argue that additional mechanisms besides education can establish tolerance to missing-self as previously suggested (Shifrin et al., 2016).

Although many studies have shown that NK cell education correlates with expression of self-MHC-I-specific Ly49 receptors (Fernandez et al., 2005; Kim et al., 2005), it has been challenging to study the mechanism of education due to a lack of Ly49 mutant mice. Here we show with the AYF mouse that Ly49 signaling is required for education and that the ITIM mediates both effector inhibition and education. These results argue against the possibility that Ly49s signal through an unidentified motif to educate NK cells. These results are consistent with the disarming model of NK cell education (Raulet and Vance, 2006), but it remains possible that distinct pathways downstream of the Ly49 ITIM mediate inhibition and education. Notably, *Ptpn6*$^{fl/fl}$ NKp46-Cre mice that lack SHP-1 in NK cells exhibit a defect in NK cell education and also a modest defect in NK cell maturation based on expression of CD11b and CD27 (Viant et al., 2014). In contrast, we did not observe a defect in NK cell maturation in AYF D8 KODO compared to D8 KODO mice despite the complete block in education through Ly49A. This discrepancy may suggest that the effects of SHP-1-deletion in NK cells may be influenced by the function of SHP-1 downstream of receptors other than self-MHC-I-specific Ly49s. Because our results show that Ly49 ITIM signaling is required for education, future studies will be needed to better characterize downstream mediators of Ly49 ITIM signaling in primary NK cells to differentiate between the arming and disarming models. Altogether, our results show that the Ly49-MHC-I interaction is required for acquisition and maintenance of NK cell education.

4.6 Inflammation promotes missing-self reactivity

Here we show that inflammatory stimuli induce NK cell-mediated rejection of $CD4^+$ T cells in tamoxifen-treated *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2} mice. These results show that MHC-I

downregulation *in vivo* can trigger missing-self reactivity but substantial missing-self reactivity is restricted to inflammatory settings. Cytokines such as IL-12 and IFN-I have been shown to be required for NK cell-mediated cytotoxic control of MCMV by priming NK cells to express perforin and granzyme B (Fehniger et al., 2007; Parikh et al., 2015). Similarly, we hypothesize that inflammation promotes missing-self reactivity through priming NK cells. This model suggests that NK cell tolerance to missing-self in tamoxifen-treated $B2m^{fl/fl}$ CD4-Cre-ER^{T2} mice is due to a lack of priming. These results are reminiscent of previous studies that showed that RIP-GP mice that express the LCMV glycoprotein in pancreatic beta cells do not exhibit autoimmune diabetes because of T cell ignorance (Ohashi et al., 1991; Oldstone et al., 1991). Infection of RIP-GP mice with LCMV, however, primes previously ignorant T cells to trigger autoreactivity and diabetes onset (Ohashi et al., 1991; Oldstone et al., 1991). Our results suggest that NK cell ignorance of missing-self contributes to the lack of autoreactivity in $B2m^{-/-}$ mice in addition to a lack of education.

Our findings provide an *in vivo* correlate to previous studies that showed that NK cells are only weakly cytotoxic against most target cells *in vitro* without IL-2 culture or pre-activation with poly(I:C) (Grimm et al., 1982; Liao et al., 1991). It remains unclear, however, why NK cells are able to kill adoptively transferred target cells, such as MHC-I-deficient splenocytes and mismatched bone marrow, without prior activation (Bix et al., 1991; Cudkowicz and Bennett, 1971; Oberg et al., 2004). It is possible that processing and adoptively transferring single-cell suspensions of bone marrow or splenocytes induces sufficient inflammation in recipient mice to prime NK cells to mediate missing-self recognition. We find this unlikely, however, because we also observed low-level rejection of MHC-I-deficient $CD4^+$ T cells in tamoxifen-treated $B2m^{fl/fl}$ CD4-Cre-ER^{T2} mice, which suggests that NK cell cytotoxicity is not solely an artifact of

adoptive transfer experiments. We find it more likely that basal inflammation is sufficient to prime NK cells for a low level of cytotoxicity that is enough to reject the relatively small number of adoptively transferred cells but not enough to mediate substantial autoreactivity.

4.7 Implications for NK cell immunotherapy

Inhibitory KIRs are attractive targets for tumor immunotherapy because antibody blockade is thought to mimic missing-self recognition to induce NK cell-mediated tumor killing (Daher and Rezvani, 2018). Our results, however, show that global MHC-I downregulation or loss of Ly49 signaling do not induce substantial missing-self recognition. Instead, we show that NK cells adapt to a loss of self-MHC-I because loss of inhibitory Ly49 signaling impairs NK cell education and alters the receptor repertoire. These results likely explain why anti-KIR2D checkpoint blockade was ineffective in treating patients with smoldering multiple myeloma, as was previously proposed based on patient data (Carlsten et al., 2016; Grossenbacher et al., 2017). However, our results suggest that combining anti-KIR antibodies with an inflammatory stimulus may increase the efficacy of NK cell checkpoint blockade, as others have also proposed (Ardolino et al., 2014; Vahlne et al., 2010)

4.8 Future directions

The AYF and floxed *B2m* mice provide valuable tools for studying the mechanism of NK cell education. Our results show that Ly49 ITIM signaling is required for NK cell education, but the downstream signaling pathways remain unclear. In the future, we will perform co-immunoprecipitation of Ly49A in LAKs from WT and AYF mice followed by mass spectrometry to identify proteins that bind to the Ly49A ITIM in primary NK cells through a non-biased approach. Additionally, we will breed floxed *B2m* mice that express various cell-

type-specific Cre recombinases to test whether NK cells are educated by MHC-I expression on specific cell types as has been previously proposed (Shifrin et al., 2016).

Here we have studied the response of NK cells to downregulation of H-2K^{b} and H-2D^{b}, but it remains unclear if our findings generalize to other MHC-I haplotypes. In the future we will breed *B2m*$^{fl/fl}$ R26-Cre-ER^{T2} D8 KODO and *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2} D8 KODO mice to study how NK cells respond to acute downregulation of H-2D^{d}. We expect that global downregulation of H-2D^{d} will lead to a loss of NK cell education, similar to what we observed with H-2K^{b} and H-2D^{b}. Additionally, we expect that downregulation of H-2D^{d} on CD4^{+} T cells will only lead to robust missing-self recognition in the context of inflammation. If this is found, then the H-2D^{d} background will allow us to test if inflammation-induced missing-self reactivity requires NK cell education by breeding *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2} AYF D8 KODO mice that should exhibit impaired education through Ly49A.

Although we have hypothesized that inflammation promotes missing-self reactivity by priming NK cells, it remains unclear if perforin and granzyme B are required for this form of missing-self reactivity. To test this, we will breed *Prf1*$^{-/-}$ *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2} and *Gzmb*$^{-/-}$ *B2m*$^{fl/fl}$ CD4-Cre-ER^{T2} mice that contain germline knockouts of perforin or granzyme B respectively. We hypothesize that inflammation will not induce NK cell-mediated rejection of MHC-I-deficient CD4^{+} T cells in perforin- or granzyme B-deficient mice. Additionally, we will test if inflammation is acting on the NK cell or the target cell by breeding *B2m*$^{fl/fl}$ *Ifnar1*$^{fl/fl}$ CD4-Cre-ER^{T2} mice to delete IFNAR on only the target cells and *B2m*$^{fl/fl}$ *Ifnar1*$^{fl/fl}$ NKp46-Cre CD4-Cre-ER^{T2} to delete IFNAR on both the target cells and the NK cell. We hypothesize that inflammation will induce NK cell rejection of MHC-I-deficient CD4^{+} T cells in tamoxifen-

treated *B2m*$^{fl/fl}$ *Ifnar1*$^{fl/fl}$ CD4-Cre-ER^{T2} and *B2m*$^{fl/fl}$ NKp46-Cre CD4-Cre-ER^{T2} mice but not in tamoxifen-treated *B2m*$^{fl/fl}$ *Ifnar1*$^{fl/fl}$ NKp46-Cre CD4-Cre-ER^{T2} mice.

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